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RELATION BETWEEN BIOLOGICAL ACTIVITIES IN THE PRESENCE OF VARIOUS SALTS AND THE CONCENTRATION OF THE SOIL SOLUTION IN DIFFERENT CLASSES OF SOIL[1]

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INTRODUCTION

Experiments dealing with the concentration of the soil solution in soils of different classes when treated with various salts have been in progress in this laboratory for some time, and it has now become very desirable to know the relation between the biological activities and the composition and concentration of the soil solution under these conditions.

Several workers have shown that certain salts occurring naturally in many soils and often added as fertilizers and amendments have pronounced effects on the bacterial flora present. The causes of these effects have not been entirely determined, it being assumed that both toxicity and osmotic pressure are operative.

HISTORICAL REVIEW

No attempt will be made to give a complete résumé of the literature on toxicity, but the writer wishes to call attention to a few points brought out by various investigators.

A review of the available literature on the toxicity of salts for higher and lower plant life shows considerable disagreement in the results obtained by different investigators. It is suggested by Greaves[3] that in many cases this may be explained by the unknown variation in the actual amounts of salts added, and he proposes to remedy the deficiency by analyzing the solutions used to supply the salts and correcting for the variations found.

Greaves[3] found that calcium nitrate, magnesium nitrate, calcium chlorid, sodium sulphate, and potassium sulphate were toxic to the

[1] Publication authorized by Dean R. S. Shaw, Director of the Michigan Agricultural Experiment Station.

[2] The writer wishes to express his gratitude to Dr. M. M. McCool, of the Michigan Experiment Station, for many helpful suggestions during the completion of these experiments and preparation of the manuscript.

[3] GREAVES, J. E. THE INFLUENCE OF SALTS ON THE BACTERIAL ACTIVITIES OF THE SOIL. *In* Soil Science, v. 2, no. 5, p. 443–480, 4 fig. 1916.

ammonifiers in very low concentration (78×10^{-3} mol. per 100 gm. of soil) and all the other salts used, with the exception of calcium carbonate and manganous carbonate, became toxic in some of the concentrations tested. He states (p. 474) that—

> The increased osmotic pressure exerted by the salts added to a soil plays an important part in the retarding of the bacterial activity, it is not the only nor probably the main influence. The main influence is likely to be a physiological one due to the action of the substance upon the living protoplasm of the cell, changing its chemical and physical properties so that it cannot function properly.

Osmotic pressure as the causal factor is also suggested by the work of Harris,[1] who found that the germination of different seeds was first retarded by the salts studied when the soils contained a small amount of moisture. With most of the salts the highest germination was in the wettest sand, while with sodium chlorid the medium moisture gave the highest germination.

He also states that (p. 44)—

> In a general way salts with low molecular weights are more toxic than those having a higher molecular weight, but there are so many exceptions that this can not be considered a general law holding for all salts.

That the soil plays an important rôle in the toxic action of salts was shown by Harris,[1] who points out (p. 26) that—

> Plants were able to endure much stronger chlorids and nitrates in solution culture than in the soil, while the carbonates retarded growth more in the solution than in the loam, but not as much as in the sand.

The antagonism of salts was found to be less in soils than in solutions. He also concludes that only about one-half as much alkali is required to prohibit the growth of crops in sand as in loam soil. Sodium carbonate was more toxic in sand than sodium chlorid, while with loam the reverse was true.

That soils of different chemical and physical properties influence differently the effect of added substances on the lower organisms is shown by the work of Lipman and Burgess[3] when they found that the order of nitrifiability of various nitrogenous substances depends largely on the soil used.

Further evidence of the importance of the interaction of the soil with the salts added is brought out by Headley, Curtis, and Scofield[4] in determining the relative toxicity of sodium carbonate, sodium bicarbonate, sodium chlorid, and sodium sulphate for wheat. They not only

[1] Harris, F. S. Effect of alkali salts in soils on the germination and growth of crops. *In* Jour. Agr. Research, v. 5, no. 1, p. 1–53, 48 fig. 1915. Literature cited, p. 52–53.

[3] Lipman, C. B., and Burgess, P. S. The determination of availability of nitrogenous fertilizers in various California soil types by their nitrifiability. Cal. Agr. Exp. Sta. Bul. 260, p. 103–127. 1915.

[4] Headley, F. B., Curtis, E. W., and Scofield, C. S. Effect on plant growth of sodium salts in the soil. *In* Jour. Agr. Research, v. 6, no. 22, p. 857–869, 8 fig. 1916.

measured the amount of the salt added but at the completion of the period of growth extracted the soil with water and determined the quantity of salt removable. They arrived at the following conclusion (p. 869):

> The limit of tolerance of crop plants to the salt in the soil is determined by the quantity of salt that can be recovered from the soil rather than by the quantity added to the soil.

The toxicity of sodium carbonate was much greater in beach sand than in loam soil, and the loss of carbonate was much less in the sand than in the loam.

Bouyoucos and McCool [1] studied more in detail the action of salts in soils and found that equal amounts of various salts added to different soils result in widely different concentrations of the soil solution and suggest that there is probably considerable change in the chemical composition of the resulting soil solution.

More definite information regarding the difference in end products formed when salts come in contact with different soils is furnished by McCool and Wheeting,[2] who show that the amounts of water-soluble calcium, iron, and magnesium in soils through which various salts have diffused are quite different and that the quantities of these elements in the layers of soil at various distances from the salt deposit are variable.

This interaction of soil and salt has seemingly been overlooked by some investigators in accounting for the toxic action of salts; and it was with the hope of obtaining more evidence as to whether osmotic pressure, the toxic properties of the salt itself, or the products of the interaction of the soil and salt was responsible for the effects noted that the following experiments were conducted.

EXPERIMENTAL WORK.

Three soils were used in this work: A Coloma sand, a Miami sandy loam, and a Clyde clay loam. The soils were brought to the laboratory, dried, passed through a 20-mesh sieve, mixed thoroughly, and stored in the dark. As needed, 100-gm. samples were weighed into sterile, covered tumblers and thoroughly mixed with 2 gm. of finely ground dried blood. The salts were applied in solution. The most concentrated solution was made by weighing out the correct amount of the salt and dissolving it in distilled water. The solution of next lower concentration was made by drawing off a portion of the first solution and making it up to the required volume by weight. The third solution was made from the second in a similar manner. The solutions were

[1] BOUYOUCOS, G. J., and MCCOOL, M. M. THE FREEZING-POINT METHOD AS A MEANS OF MEASURING THE CONCENTRATION OF THE SOIL SOLUTION DIRECTLY IN THE SOIL. Mich. Agr. Exp. Sta. Tech. Bul. 24, p. 592–631, 2 fig. 1916.

[2] MCCOOL, M. M., and WHEETING, L. C. MOVEMENT OF SOLUBLE SALTS THROUGH SOILS. *In* Jour. Agr. Research, v. 11, no. 11, p. 531–547, 5 fig. 1917.

sterilized, a small quantity was poured into a sterile container, and the desired amount was drawn off in a sterile pipette graduated to 0.01 c. c. The solution was mixed with enough sterile water to make the soil up to optimum moisture content, and the liquid was thoroughly mixed through the soil with a sterile spatula. The samples were incubated for four days at 29° to 30° C. and then distilled from copper flasks with magnesium oxid in the usual way. The data presented represent the average of three or more determinations which agree within 12 per cent and with a few exceptions within 10 per cent.

The freezing-point determinations were made by mixing 25 gm. of soil with a proportional amount of solution, after which the mixture was placed in a freezing tube and the freezing point determined with a Beckman thermometer. The data given represent the average of two determinations.

The following tables (I–VII) show the amount of the salt added, the nitrogen produced from 2 gm. of dried blood, the depression of the freezing point of the soil solution, and the corresponding osmotic pressure.

While working with the sandy loam, it became apparent to the writer that the increments by which the amounts of the salt used in the various tests increased were much smaller than necessary to bring out the points desired, and consequently some of the concentrations were omitted in the clay-loam series.

TABLE I.—*Ammonification of dried blood in sandy loam and clay loam with varying percentages of magnesium sulphate*

Salt in air-dry soil.	Sandy loam.			Clay loam.		
	Nitrogen produced.	Depression of freezing point.	Osmotic pressure.	Nitrogen produced.	Depression of freezing point.	Osmotic pressure.
Per cent.	*Mgm.*	°C.	*Atmospheres.*	*Mgm.*	°C.	*Atmospheres.*
0.000	75.70	0.285	3.435	52.10	0.276	3.327
.001	69.67	.289	3.483	56.36	.288	3.471
.002	71.91	.275	3.315	.....	.....	.....
.008	76.12	.280	3.375	58.60	.290	3.496
.016	77.38	.308	3.712	56.36	.315	3.797
.032	76.54	.280	3.375	57.20	.288	3.471
.064	68.97	.330	3.978	53.84	.308	3.712
.100	67.57	.333	4.014	52.02	.303	3.652
.200	61.87	.403	4.857	.....	.....	.....
.300	62.67	.462	5.567	49.64	.373	4.495
.400	68.41	.530	6.386	.....	.....	.....
.500	69.25	.568	6.843	44.87	.455	5.483
.600	61.41	.610	7.349	.....	.....	.....
.700	54.82	.725	8.732	48.52	.483	5.820

The data in Table I show that for sandy loam the addition of 0.001 and 0.002 per cent of the salt resulted in a slightly lower ammonification than the untreated sample. The differences, however, are within the limits of experimental error. Concentrations of 0.008, 0.016, and 0.032

per cent of the salt gave results practically identical with those of the untreated soil; but with an addition of 0.064 per cent a marked decrease in ammonia production resulted. With a higher percentage of the salt the results show irregularities due to experimental errors, but they also show a general tendency to depress ammonification still further. When 0.7 per cent of the salt was added, a very great decrease resulted in the amount of ammonia produced.

A tendency to increase the osmotic pressure in the sandy-loam series is noticeable with an addition of 0.016 per cent of the salt. This tendency, however, is more pronounced at 0.064 per cent; and thereafter the osmotic pressure rises steadily with no irregularities.

The results with clay loam show a small but persistent stimulation of the ammonifying organisms until a concentration of 0.064 per cent of the salt is reached, when the production of ammonia drops back to practically that of the untreated soil.

There is very little further decrease in the amount of ammonia produced with a greater application of the salt, except in the case of 0.500 per cent. Since, however, the production with 0.700 per cent rises to practically that of the untreated soil, it seems probable that the reduction with 0.500 per cent of the salt is an experimental error. There is no point, therefore, at which it can be said that a marked toxicity to ammonification occurs.

It is interesting to note that there is no marked increase in the osmotic pressure of the soil solution until an addition of 0.300 per cent of the salt has been made, which is somewhat more than the amount present when ammonification was reduced to normal.

TABLE II.—*Ammonification of dried blood in sandy loam and clay loam with varying percentages of calcium nitrate*

Salt in air-dry soil.	Sandy loam.			Clay loam.		
	Nitrogen produced.	Depression of freezing point.	Osmotic pressure.	Nitrogen produced.	Depression of freezing point.	Osmotic pressure.
Per cent.	*Mgm.*	°C.	*Atmospheres.*	*Mgm.*	°C.	*Atmospheres.*
0.000	75.70	0.285	3.435	52.10	0.276	3.327
.001	76.68	.263	3.170	55.52	.275	3.315
.002	75.56	.280	3.375	.....	.....	.....
.008	77.38	.252	3.038	55.24	.285	3.435
.016	74.72	.277	3.339	51.88	.295	3.546
.032	75.70	.307	3.700	49.92	.290	3.496
.064	73.59	.364	4.387	55.66	.330	3.978
.100	71.35	.394	4.748	53.70	.393	4.736
.200	64.49	.523	6.302	.....	.....	.....
.300	58.18	.685	8.251	48.24	.568	6.843
.400	56.08	.790	9.514	.....	.....	.....
.500	54.82	.930	11.20	49.36	.683	8.227
.600	50.89	1.060	12.76	.....	.....	.....
.700	48.04	1.152	13.864	47.54	.873	10.516

A consideration of Table II shows there is no apparent stimulation or depression of ammonification in sandy loam until a concentration of 0.200 per cent of calcium nitrate is reached, when a marked depression occurs, and is followed by further slight depressions with each succeeding addition of the salt. The freezing-point determinations show a well-defined increase in the osmotic pressure of the soil solution when 0.032 per cent of the salt is added, with a further increase with each succeeding increase in the amount of calcium nitrate added.

With clay loam there is apparently no stimulation of the bacterial activities such as occurred in the case of magnesium sulphate. The two series are similar, however, in that there is no point at which the salt exhibits a marked toxicity such as is seen in the case of sandy loam.

The osmotic pressure of the soil solution is decidedly increased when 0.064 per cent of calcium nitrate is present, and steadily rises as more of the salt is added.

TABLE III.—*Ammonification of dried blood in sandy loam and clay loam with varying percentages of calcium chlorid*

Salt in air-dry soil.	Sandy loam.			Clay loam.		
	Nitrogen produced.	Depression of freezing point.	Osmotic pressure.	Nitrogen produced.	Depression of freezing point.	Osmotic pressure.
Per cent.	*Mgm.*	*°C.*	*Atmospheres.*	*Mgm.*	*°C.*	*Atmospheres.*
0.000	75.70	0.285	3.435	52.10	0.276	3.327
.001	78.22	.280	3.375	46.13	.280	3.375
.002	79.76	.290	3.496	.....	.....	.....
.008	80.18	.280	3.375	43.19	.300	3.616
.016	75.84	.316	3.809	42.21	.315	3.797
.032	73.59	.353	4.255	50.90	.320	3.857
.064	66.17	.450	5.423	55.10	.430	5.182
.100	60.43	.523	6.302	49.22	.470	5.664
.200	59.72	.708	8.528	.....	.....	.....
.300	59.02	1.068	12.856	47.12	.810	9.755
.400	59.86	1.385	16.660	.....	.....	.....
.500	52.30	1.620	19.48	44.31	1.163	13.996
.600	45.99	1.885	22.660	.....	.....	.....
.700	43.05	.....	.....	45.29	1.455	17.500

When calcium chlorid is added to sandy loam, we again see (Table III) an apparent slight stimulation of ammonification with 0.001, 0.002, and 0.008 per cent of the salt; but when 0.016 per cent is added, the process is reduced to normal; and with 0.064 per cent there occurs a marked depression. With further addition of the salt, ammonification is depressed as a whole, but the results are irregular.

The freezing points of the soil show that an increase of osmotic pressure occurred when 0.016 per cent of calcium chlorid was added, which agrees with the point brought out in Table II—namely, that an increase in osmotic pressure of the soil solution is observed with amounts of salts

which do not depress ammonification. It is rather striking in this series, however, that when an increase in osmotic pressure occurs the ammonia produced drops to that of the untreated soil.

The data with clay loam are exceedingly interesting. There appears at first to be a slight depression of ammonia production, the depression increasing until with 0.016 per cent of calcium chlorid it reaches almost 9 mgm. of nitrogen, or 19 per cent, considering the production in the untreated soil as 100 per cent. With 0.032 per cent of salt ammonification returns to normal and remains so until 0.300 per cent of salt is present, when it is slightly depressed again and remains so with but small increases of depression throughout the series. In no other concentration in the series, however, is the ammonia production reduced to quite so low a point as with 0.008 and 0.016 per cent of the salt.

The osmotic pressure of the soil solution shows a sharp increase with addition of 0.064 per cent of calcium chlorid, and thereafter there is a further increase with each addition of the salt. It is noteworthy, however, that ammonification returns to normal before there is any appreciable increase in the concentration of the soil solution.

TABLE IV.—*Ammonification of dried blood in sandy loam and clay loam with varying percentages of potassium chlorid*

Salt in air-dry soil.	Sandy loam.			Clay loam.		
	Nitrogen produced.	Depression of freezing point.	Osmotic pressure.	Nitrogen produced.	Depression of freezing point.	Osmotic pressure.
Per cent.	*Mgm.*	*°C.*	*Atmospheres.*	*Mgm.*	*°C.*	*Atmospheres.*
0.000	75.70	0.285	3.435	52.10	0.276	3.327
.001	71.63	.280	3.375	50.08	.260	3.134
.002	73.87	.273	3.291	.....	.....	.....
.008	73.45	.298	3.592	45.85	.276	3.327
.016	69.53	.325	3.917	49.08	.305	3.676
.032	67.99	.373	4.495	48.38	.302	3.640
.064	60.43	.440	5.302	50.48	.336	4.050
.100	57.20	.538	6.482	45.71	.421	5.074
.200	54.26	.858	10.336	.....	.....	.....
.300	63.65	1.118	10.768	38.29	.765	9.214
.400	57.06	1.410	16.960	.....	.....	.....
.500	52.44	1.723	20.716	38.43	1.093	13.156
.600	44.03	.....	.....	.....	.....	.....
.700	33.95	.....	.....	35.07	1.455	17.500

The data in Table IV show a well-defined decrease in ammonification when 0.064 per cent of potassium chlorid is added to sandy loam; however, a decided increase in the osmotic pressure of the soil solution is noticeable when only one-fourth of this amount of the salt is added. This agrees closely with the results obtained when calcium chlorid and calcium nitrate are applied to this soil.

With clay loam the results are quite uniform until 0.300 per cent of salt is added, when there occurs a sharp decrease in the amount of ammonia produced. This is the only salt tested which gives results of this kind with clay loam. With the other salts the decrease in ammonification is gradual, no special point being observed where a marked decrease in ammonification occurs. As in the results with sandy loam, an increase in the osmotic pressure of the soil solution is observed somewhat before any decrease in bacterial activities are evident.

GENERAL DISCUSSION

The data presented bring out, first of all, that there is a decided difference in the effect on ammonification of the salts when applied to different soils. With every salt tested there was found with sandy loam a point at which marked depression of the ammonifying power occurred, while with clay loam such a point was found for potassium chlorid only. Whether this difference is due to a difference in the flora present or to the physical or chemical properties of the soil is undetermined. In the light of the work of Bouyoucos and McCool [1] and McCool and Wheeting,[2] however, there is unquestionably a difference in the composition of the soil solution. The difference in the behavior of these soils emphasizes the importance of much thorough investigation of the problem of toxicity of salts in soils.

Another point of interest is the lack of regularity in the results after a point is reached where ammonification is markedly depressed. It might be expected, whether the depression is due to toxicity or osmotic pressure, that from this point every addition of salt would cause an ever-increasing amount of depression. In no case, however, is this found to be true. Where there is a consistent decrease in ammonia production after this critical point is passed, such as occurs with calcium nitrate, the decreases are small. In many cases there are fluctuations, such as occur with potassium chlorid and magnesium sulphate, which are generally ascribed to experimental error. Again there are cases where further additions of salt fail to exert any further depressing effect on the ammonifying organisms, even though the amount of salt added may be increased four times, as is seen in the tests with calcium chlorid. It seems when such results as referred to above are obtained that there must be some fundamental cause for the variation, a possible explanation being complex chemical interchanges between the salts and the soil constituents, the end products depending on the amount of salt present. That some such factor is operative is made more evident by such results as those

[1] Bouyoucos, G. J., and McCool, M. M. Op. cit.
[2] McCool, M. M., and Wheeting, L. C. Op. cit.

obtained when calcium chlorid is added to clay loam; and in fact all the data from clay loam indicate the operation of some influence as yet not understood.

That the soil used has an important bearing on the results to be expected is made further evident by a comparison of the amounts of the different salts needed to bring about changes in the osmotic pressure of the soil solution and also in the depression of ammonification. A summary of results showing these points is given in Table V.

TABLE V.—*Percentage of salts and the resulting osmotic pressures at which depression of ammonification and increase in osmotic pressure of the soil solution are observed.*

Salt used.	Sandy loam.				Clay loam.			
	Depression of ammonification.		Increase in osmotic pressure.		Depression of ammonification.		Increase in osmotic pressure.	
	Salt.	Osmotic pressure.	Salt.	Osmotic pressure.	Salt.	Osmotic pressure.	Salt.	Osmotic pressure.
	Per cent.	*Atmospheres.*	*Per cent.*	*Atmospheres.*	*Per cent.*	*Atmospheres.*	*Per cent.*	*Atmospheres.*
Magnesium sulphate	0.064	3.978	0.016	3.712	.....	.....	.....	.....
Calcium nitrate	.200	6.302	.032	3.700	.....	.....	0.064	3.978
Calcium chlorid	.064	5.423	.016	3.809	.....	.....	.064	5.182
Potassium chlorid	.064	5.302	.016	3.917	0.300	9.214	.064	4.050

These data show that four times as much of the salt is required to produce a noticeable increase in the osmotic pressure of the soil solution in clay loam as in sandy loam when calcium chlorid and potassium chlorid are added. In the case of calcium nitrate only twice as much is required, while with magnesium sulphate 19 times as much is necessary. It is also noticeable that in sandy loam depression of ammonification occurs with the same amount of each salt except where calcium nitrate is used, when a greater amount is also needed to increase the osmotic pressure of the soil solution.

There is considerable variation in the osmotic pressure at which ammonification is depressed in sandy loam. The osmotic pressure of the soil solution in clay loam when ammonification is decreased by potassium chlorid is much greater than the osmotic pressure in sandy loam when depression occurs as the result of additions of any salt.

It appears from these data that the decrease in ammonification resulting from the addition of the salts used is not due to a change in osmotic pressure and that the soil itself is a very potent factor in determining the effect.

In order to judge more accurately the part played by osmotic pressure in modifying the action of ammonifying organisms a series was run with potassium chlorid in sand. The results are given in Table VI.

TABLE VI.—*Ammonification of dried blood in sand with varying percentages of potassium chlorid*

Salt in air-dry soil.	Sand.		
	Nitrogen produced.	Depression of freezing point.	Osmotic pressure.
Per cent.	*Mgm.*	*°C.*	*Atmospheres.*
0.000	32.64	1.157	13.924
.001	33.34	1.109	13.348
.002	31.80	1.120	13.48
.004	32.36	1.137	13.684
.008	24.73	1.101	13.372
.016	29.14	1.086	13.072
.032	28.44	1.160	13.96
.064	23.82	1.587	19.084
.100	27.88	1.837	22.084
.200	16.53	2.746	32.962
.300	10.09	.....	.....
.400	6.44	.....	.....
.500	5.88	.....	.....

These results show ammonification proceeding normally when the osmotic pressure of the soil solution is from 13 to 14 atmospheres. This is twice the osmotic pressure in sandy loam when calcium nitrate depressed ammonification and three times that when magnesium sulphate caused a decrease in ammonia production. It seems justifiable from the data presented to conclude that osmotic pressure is not the determining factor in the reduction of ammonification by the salts used.

One point should be mentioned in this connection. Neither in this work nor in the results of other workers reviewed by the writer have the total osmotic pressures of the soil solution been reported at the time the ammonia determinations were made. It is possible that the pressure due to the products of bacterial action plus that due to the salt added may equal a constant.

TABLE VII.—*Effect of dried blood on the osmotic pressure of the soil solution*

Kind of soil.	Amount of soil.	Amount of dried blood.	Freezing-point depression.	Osmotic pressure.	Difference in osmotic pressure due to dried blood.
	Gm.	*Gm.*	*°C.*	*Atmospheres.*	*Atmospheres.*
Sand	100	0.0	0.160	1.930	.....
Do	100	1.0	.267	3.218	1.288
Do	100	2.0	1.157	13.924	11.994
Sandy loam	100	.0	.135	1.518	.....
Do	100	1.0	.190	2.291	.773
Do	160	2.0	.285	3.435	1.917
Clay loam	100	.0	.160	1.930	.....
Do	100	1.0	.195	2.351	.421
Do	100	2.0	.276	3.327	1.397

One point which, so far as the writer is informed, has been omitted in toxicity work is the increase of osmotic pressure due to the addition of dried blood. Table VII shows the increase in the osmotic pressure of the soil solution of the soils used in the above experiments due to the addition of various amounts of dried blood.

It is very evident that the amount of blood used in ammonifying tests has a great influence on the concentration of the soil solution and that the increase in osmotic pressure is quite different with various classes of soils. Just what effect the addition of varying amounts of such material will have on the ammonifying power of the soil is yet to be determined. It seems improbable that results obtained under such abnormal conditions will represent the power of soils to ammonify nitrogenous materials under field conditions where much lighter applications are made.

CONCLUSIONS

Since the osmotic pressure at which ammonification of dried blood is depressed in sandy loam by the addition of various salts is different for each salt tested, it seems improbable that osmotic pressure is the governing factor. This conclusion is further strengthened by the observation that ammonification proceeds unimpaired in sand where the osmotic pressure of the soil solution is between 13 and 14 atmospheres, while in sandy loam the process is depressed when the osmotic pressure reaches 4 to 6 atmospheres and in clay loam at a pressure of about 9 atmospheres.

The effect of various salts on ammonification is apparently modified very materially by the nature of the soil used. Thus, for the four salts studied, each gave a definite point where the ammonification of dried blood in sandy loam was depressed, while only one salt gave such a point with clay loam. The cause of such variations is yet to be investigated, but it seems possible that the chemical reaction between the salt added and the soil constituents may play some part.

The addition of the amount of dried blood usually used in ammonification work has a very appreciable effect on the osmotic pressure of the soil solution, the increase varying with the class of soil used.

The effect of various salts on the ammonification of nitrogenous materials in soils offers the opportunity for much thorough investigation, one of the chief needs being improved methods.

BACTERIAL FLORA OF ROQUEFORT CHEESE

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INTRODUCTION

It is well known that various kinds of hard cheese, such as Cheddar and Emmental, or Swiss, depend on a suitable bacterial flora for their normal development. The two types of cheese mentioned have been studied extensively by a number of investigators. It has been shown that in the manufacture of Cheddar cheese there must be a rapid development of the lactic-acid bacterium *Streptococcus lacticus*, and that during the ripening a development of other forms of cocci and lactic bacteria of the *bulgaricum*[1] type is necessary to produce the typical Cheddar flavor (*4*).[2] In Swiss-cheese manufacture the *Bacterium bulgaricum* is added, either in pure culture, as a starter, or unwittingly, with the rennet. What other microorganisms are responsible for the characteristic sweetish flavor, and for the development of the "eyes"; has not yet been established, but at any rate the ripening is due to the growth of bacteria, in distinction from the mold-ripened cheeses.

It is obvious that in cheeses which are mottled with molds the molds play an important part in the ripening. The mold which ripens Roquefort cheese, and also Gorgonzola and Stilton cheeses, has been named "*Penicillium roqueforti* (Thom)" (*6*). Currie (*1*) has shown that it hydrolyzes fat and the resulting acids have the peppery or burning effect on the tongue and palate which is characteristic of Roquefort cheese.

In the making of Roquefort cheese, as in the making of Cheddar cheese, a rapid development of lactic-acid bacteria is necessary to bring about the proper physical condition of the curd in the various stages of manufacture. This acidity results from the growth of *Streptococcus lacticus*. The importance of the *S. lacticus* and *Penicillium roqueforti* in the making and ripening of Roquefort cheese was recognized by Thom, who

[1] In earlier publications on the flora of Cheddar cheese these organisms were called "*Bacterium casei*," the name which Freudenreich applied to the lactic-acid-producing rod forms of Emmental cheese. He failed to recognize the organisms as similar to the one which he had isolated from kefir, and which he called "*Bacterium caucasicum*"; later it received the name "*bulgaricum*." There does not appear to be sufficient cultural, morphological, and chemical differences between the lactic-acid-producing rod forms from cheese and from other sources to justify the use of two species names, although the strains isolated from cheese are more hardy than those isolated from oriental milk drinks in respect to growth at low temperatures and in respect to growth on ordinary media. In this paper the species will be designated as "*bulgaricum*," the term accepted by common usuage.

[2] Reference is made by number (italic) to "Literature cited," p. 233.

made a study of mold-ripened cheeses. His observations (*6*) led him to believe that those two microorganisms were—

> capable of ripening Roquefort cheese without the introduction of other enzyme producing or flavor producing organisms.

In the Dairy Division experimental work, when cheese of the Roquefort type was obtained, the question was raised as to whether the bacterial flora had anything to do with the difficulties which arose; whether cheese made from sheep's milk, according to the Roquefort way in France, differed significantly in its bacterial flora from cheese made in a similar manner from cows' milk in America. Accordingly, a study of the bacterial flora of Roquefort cheese, imported and experimental, was undertaken.

METHODS OF CHEESE EXAMINATION

The method of examination was similar to that used in the bacteriological analyses of Cheddar cheese (*4*). Plate cultures were made on infusion agar, and for comparison numerous colonies were "fished off" into litmus skim milk. Representative cultures of every type of organism appearing in considerable numbers were saved for detailed study.

Cultures for study were also obtained by the following method: Milk cultures were inoculated from dilutions of a cheese emulsion, the dilution increasing from tube to tube by a ratio of 10. The dilutions ranged from 1 to 10 to 1 to 1,000,000,000. The lower dilutions were inoculated into tubes which had been modified as follows to separate the mold growth from the bacterial growth: Ordinary test tubes were drawn out to form a constriction beginning about 4 cm. from the bottom of the tube and filled with skim milk to about 2 cm. above the top of the constriction. Since oxygen is required for mold growth, the part of the culture below the constriction was always free from molds. By breaking the tube at the constriction it was possible to obtain a subculture of bacteria free from molds. As soon as a milk culture in any dilution showed evidence of bacterial growth, it was plated out to obtain a pure culture of the predominating organism. Many of the cultures thus obtained duplicated the cultures obtained by the plate method, but sometimes an organism was obtained which would be missed if plate cultures alone were made.

The pure cultures were studied morphologically and biochemically. The detailed study of the different groups of organisms will be presented in separate papers, for the bacteria of Roquefort cheese are not of types peculiar to that kind of cheese, but are ripening agents common to many other kinds, each species differing in importance in different kinds of cheese, according to the various conditions to which it is subjected.

BACTERIAL FLORA OF IMPORTED ROQUEFORT CHEESE

Bacteriological analyses of a number of imported Roquefort cheese have been made and the data are presented in Table I. Most of the imported cheese obtained for study was well ripened when the analysis was made. Cheeses 13, 146, 148, and 150 were not well ripened, but they must have been several weeks old, for *Streptococcus lacticus*, which certainly must have been present in the cheesemaking, had disappeared entirely from every sample examined.

TABLE I.—*The bacterial flora of imported Roquefort cheese*

Cheese No.	Number of organisms per gram of cheese.		Cheese No.	Number of organisms per gram of cheese.	
	Cheese streptococci.	*Bacterium bulgaricum.*		Cheese streptococci.	*Bacterium bulgaricum.*
13	31,000,000	100,000	137	600,000	1,000,000
15	600,000	400,000	139	10,000	1,000
17	13,000,000	4,000,000	141	100,000,000	1,000,000
18	30,000,000	33,000,000	143	1,000,000	1,000,000
131	1,400,000	145,000	146	210,000	250,000
133	17,000,000	.....	148	1,000,000	24,000,000
135	140,000,000	4,000,000	150	1,000	10,000

Two groups of bacteria were found to be the common flora of this type of ripened cheese: *Bact. bulgaricum*, and a group of organisms which here will be called "cheese streptococci." [1]

Table I shows that cheese streptococci in varying numbers, with 140,000,000 per gram as the highest number, were isolated from every one of the imported cheeses, and *Bact. bulgaricum* was isolated from all but one of them, with 33,000,000 per gram as the highest number. It most probably was present in the one cheese also, but failed to be isolated. Yeasts were isolated from a few of the cheeses, but in too small numbers to be considered of any significance.

BACTERIAL FLORA OF EXPERIMENTAL CHEESE MADE ACCORDING TO THE ROQUEFORT METHOD

For several years experimental cheese has been made in the Dairy Division according to the method by which Roquefort cheese is made, but with cows' milk instead of sheep's milk. The ripened cheese is very similar to the imported variety in appearance and flavor. The data obtained from a detailed study of the bacterial flora of the experimental cheese are given in Table II.

[1] The cheese streptococci are the subject of an accompanying paper (3). Culturally they are distinguished from *Streptococcus lacticus* by a failure to curdle litmus milk with the reduction of the litmus characteristic for that organism.

TABLE II.—*Bacterial flora of cheese made in the dairy division according to the Roquefort method. Number of organisms per gram of cheese*

Cheese No.	Age.	*Streptococcus lacticus.*	Cheese streptococci.	*Bacterium bulgaricum.*	Yeast.
1732...	These four samples were taken at progressive stages of the cheesemaking.	10, 000, 000	.....	.....	.....
1732...		20, 000, 000	.....	10	.....
1732...		6, 500, 000	.....	.....	.....
1732...		9, 000, 000	.....	.....	.....
1732...	1 day	100, 000, 000	.....	.....	.....
1732...	2 days	10, 000, 000	100, 000, 000	.....	.....
96. 115.	3 days	10, 000	5, 000, 000	.....	.....
1732...	4 days	1, 000, 000	100, 000, 000	.....	.....
1732...	6 days	1, 000, 000	.....	.....	.....
1732...	8 days	100, 000	100, 000, 000	.....	.....
1732...	12 days	100, 000	31, 000, 000	.....	300
1732...	16 days	.....	.....	10, 000	1, 000
1732...	23 days	100, 000	10, 000, 000	.....	.....
1789...	25 days	.....	7, 500, 000	14, 000, 000	100, 000
1754...	35 days	.....	1, 000, 000	.....	1, 000, 000
1727...	38 days	.....	120, 000	.....	.....
96. 121.	44 days	.....	1, 000, 000	.....	400, 000
96. 17..	48 days	.....	40, 000	100	100, 000
96. 124.	2½ months	.....	10, 000	1, 000, 000	10, 000, 000
96. 125.	3 months	.....	100, 000	.....	2, 500, 000
1621...	3⅓ months	.....	.....	100, 000	40, 000
1601...	3¾ months	.....	.....	.....	1, 000
1590...	4 months	.....	1, 000, 000	470, 000	.....
96. 12..	4½ months	.....	2, 150, 000	1, 000	.....
96. 113.	6 months	.....	10, 000, 000	1, 000	.....
96. 129.	6⅓ months	.....	170, 000	.....	.....

During the manufacturing of the cheese the *Streptococcus lacticus* which had been added as a starter was the only organism present in sufficient numbers to appear on the plates. These organisms gradually disappeared, and on the twenty-fifth day their numbers had so diminished that they no longer appeared on the plates.

A normal Roquefort cheese contains 4 per cent of sodium chlorid (NaCl) in 40 per cent of water, which can be regarded as a 10 per cent brine (*8*). An experiment was carried out to show the effect of a high concentration of salt on *Streptococcus lacticus*. Flasks of milk were inoculated with *S. lacticus*, incubated at 30° C. for about five hours, when sodium chlorid was added to make a concentration of 10 per cent. In five days all the organisms had been injured so that inoculations into tubes of litmus milk gave no growth. Later, a hardier strain of *S. lacticus* treated in the same manner was killed by the 10 per cent salt solution between the tenth and the seventeenth days. This experiment explains the complete disappearance of *S. lacticus* from Roquefort cheese during the first few weeks of ripening.

In the ripening cheese the cheese streptococci had multiplied to 100,000,000 per gram on the second day. A 6-months-old cheese still contained 10,000,000 of them, which demonstrates their hardiness in respect to a high concentration of the salt, quite unlike the sensitiveness of *S. lacticus*.

In the sample tested it was found that there were 10 cells of *Bact. bulgaricum* per gram of the curd just before cutting. By the twenty-fifth day the organism had multiplied to 14,000,000 per gram. Multiplication must have continued after the cheese was salted, on the sixth and eighth days. Later, this organism appeared in varying numbers. *Bacterium bulgaricum* is thus shown to be much more hardy than *Streptococcus lacticus* in respect to a high concentration of salt.

Yeasts were found more consistently in the cheese made in the Dairy Division than in the imported cheese, one cheese containing 10,000,000 per gram. The yeasts were either species of Saccharomyces which gave a gassy fermentation in dextrose, saccharose, and lactose, but not in maltose, or they were species which fermented dextrose alone.

In a general way it can be said that the bacterial flora of the cheese from the two sources was surprisingly alike. Fifty-three cultures of cheese streptococci isolated from domestic Roquefort cheese were subjected to many biochemical tests, and if slight differences in regard to fermentable substances were considered, they could be divided into seven strains. Forty-eight cultures isolated from the imported cheese were distributed among the same seven strains, with one additional strain. The case was the same with the *Bacterium bulgaricum*. They could be divided into many strains on the basis of their fermentation reactions, but no strain was peculiar to one or the other source.

When compared with the bacterial flora of Cheddar cheese, the kinds of bacteria are the same, but the number of bacteria in Roquefort cheese is decidedly lower. Roughly it may be said that there are about one-fifth as many in Roquefort as there are in Cheddar cheese. The flora is also less constant in Roquefort cheese. Tables I and II show that occasionally from both the imported and the experimental cheese a sample which had only a few thousand bacteria per gram was found.

Unlike Cheddar cheese, the number of bacteria in Roquefort cheese appeared to have no influence upon the ripening. For example, in Table II cheese 1601 had so few bacteria that there was no growth in the cultures inoculated with the 1 to 1,000 dilution. Nevertheless it was a good cheese, with a fairly well-developed flavor. This suggests that the bacteria, with the exception of *Streptococcus lacticus*, play a very insignificant part in the ripening of Roquefort cheese, an idea that is supported by the fact that if any cheese, or any part of a cheese, fails to develop the mold, ripening takes place exceedingly slowly, but the curd remains hard and retains the acid flavor resulting from the decomposition of the lactose by *S. lacticus*, and the Roquefort flavor does not develop.

The only condition in the Roquefort-cheese curd detrimental to the activity of bacteria is the high concentration of the salt. The comparatively low numbers of bacteria are undoubtedly due to the salt, and quite probably the activity of those which are able to exist in the cheese is restrained by the salt. So far as the development of flavor is con-

cerned, it appears to be a matter of indifference whether the bacteria are present or not, because the flavor produced by *Penicillium roqueforti* is so strong that it would mask any delicate flavors produced by the bacteria. Probably the flavor substances produced by bacteria, the acids, alcohols, and esters, are consumed by the mold. This study therefore confirms Thom's (6) opinion that the only bacteria essential to the making and ripening of Roquefort cheese is the *Streptococcus lacticus*.

INFLUENCE OF SLIME ON THE RIPENING PROCESS.

There is another problem in connection with the biology of Roquefort cheese ripening—viz, the influence which the organisms in the slime may have on the ripening process. Thom and Matheson (7) paraffined a large number of experimental Roquefort cheeses before slime had opportunity to develop, and they concluded that slime is not an essential factor in flavor production, but that it serves as an index of hygrometric conditions.

In the course of this study of the biology of Roquefort cheese ripening no evidence of any ripening changes proceeding from the exterior has been observed. When the cheese is cut through at any stage of the ripening, the appearance of the cut surface is uniform and no flavors have been observed in the outside layers more pronounced than in the interior. There is always a collection of moisture on the inside of the tin foil with which Roquefort cheese is covered—explained by the fact that the interior of the cheese has a higher temperature, owing to the fermentative processes, than the outside temperature of 7° to 10° C. at which the cheese is ripened. The condensation of moisture at the surface would be followed by a return movement toward the center to maintain the moisture equilibrium. This circulatory movement would tend to distribute through the cheese the enzyms which might be liberated by the organisms growing on the surface. This, it is argued by those who believe that the slime organisms are essential to Roquefort-cheese ripening, would bring about a uniform ripening throughout the cheese mass.

As a matter of general interest, the organisms making up the slime were studied. Immediately after the manufacture of the cheese *Oidium lactis* began to grow on the surface, and by the time the cheese was 2 days old it was well covered with oidium. On the sixth day the cheese was salted and the oidium was destroyed by the salt. Then came a growth of *Penicillium roqueforti* on the surface, which gave way to the growth of the typical reddish slime.

Smears were made of the slime from the surface of imported cheese and from the surface of experimental cheese in various stages of ripening. Microscopic examination showed that the slime was made up chiefly of bacteria, with scattered cells of yeasts, but in the smears from some of the cheeses yeast cells appeared in masses. Fragments of mycelium were occasionally seen. The bacteria in every smear were a mixture of rod

and coccus forms. The organisms that made up the slime were isolated by plating on various kinds of agar, plain agar, Czapek's agar, cheese-infusion agar, and plain agar to which lactic acid was added to eliminate the growth of bacteria and to favor the growth of yeasts and molds. The isolated bacteria were submitted to various tests commonly used for the differentiation of bacteria. The most frequently isolated organisms found in the slime from the imported and the experimental cheeses was a micrococcus which gave an abundant yellowish growth on agar slope, liquefied gelatin very slightly, decomposed urea, and stopped the fermentation of carbohydrates at a hydrogen-ion concentration of about $P_H = 5.6$. Many other cocci differing only slightly from the description above were isolated from the slime. Micrococci identical with these cocci have been isolated commonly from aseptically drawn milk (*2*). It appears, then, that the udder is the source of the predominating flora of the cheese slime. There is apparently a selection of certain strains of udder cocci. The majority of them curdle milk, but no micrococcus isolated from cheese slime produced more than a slight acidity.

Another coccus fairly common in the slime from both imported and experimental cheese was exceedingly difficult to maintain on agar slopes. It formed a faint growth on agar and failed to attack the nitrogenous and carbohydrate test substances in broth cultures. It was surprising to find an organism that was capable of withstanding the rigorous conditions in the cheese slime and yet so delicate that it could scarcely be maintained under artificial cultivation.

The most frequently isolated rod form liquefied gelatin, decomposed asparagin, and gave an alkaline reaction in broths containing various carbohydrate test substances. Other rod forms differed slightly from this one.

Besides the types which have been briefly described as typical slime organisms, various kinds of cocci and rods were isolated only once or twice, which do not seem to be characteristic of the slime. For example, *Bacterium bulgaricum* was isolated from the slime once. Occasional colonies of mold, most frequently *Penicillium roqueforti*, appeared in the cultures.

None of the cheese-slime organisms were able to bring about any pronounced changes in milk in pure culture, but when pure culture of several types were inoculated together into milk their associative action digested the casein very slowly.

It would be a difficult matter to prove that these sluggish proteolytic enzyms either do or do not influence the ripening of the cheese. Gratz and Szanyi (*5*) made a careful chemical investigation of the action of the enzyms of the slime upon the ripening of the interior of hard cheeses of the Ovar and Trappist varieties on which the slime often becomes heavy. The authors concluded that in hard cheese of the type studied no ripening proceeds from the outside. The relative inactivity of the

organisms of the slime, as compared with the vigorous activity of the *Penicillium roqueforti* suggest that in Roquefort cheese also the slime organisms are of minor importance, if indeed they have any influence in the cheese ripening.

It is said that if the *Penicillium roqueforti* fails to develop, there is, nevertheless, a softening of the curd after a long period of ripening. In such case the ripening is undoubtedly brought about by the bacteria of the interior of the cheese, probably aided to a considerable extent by the enzyms from the slime; but a ripe cheese of that kind is not a typical Roquefort cheese.

The Roquefort-cheese slime is normally of a reddish color, but no organisms producing red pigment were isolated from it, although apparently all forms seen in the smears grew in the plates. The only explanation that can be offered is that the cocci, rod forms, and yeast cells, all containing more or less yellow-and-orange pigment, may produce a reddish tinge when mixed in mass. It is quite possible that the pigment production of one or all of those species is altered by the intimate association with the other species.

SUMMARY

The microorganisms essential for the manufacture and ripening of Roquefort cheese are *Streptococcus lacticus* and *Penicillium roqueforti*.

Streptococcus lacticus decomposes the lactose during the manufacture of the cheese and thus produces the lactic acid necessary for the cheese making. These organisms disappear from the cheese after about two or three weeks, being killed by the high concentration of sodium chlorid.

The remaining flora of Roquefort cheese consists of cheese streptococci and *Bacterium bulgaricum*, organisms which are found in all kinds of ripening cheese. These organisms do not have any significant part to play in the ripening of Roquefort cheese.

The cheese slime consists of characteristic types of micrococci, rod forms, and yeast cells. The enzyms from the slime do not appear to be essential to the ripening of the cheese.

The flora of both the interior and the slime of the experimental cheese was identical with the flora of the interior and the slime of the imported cheese.

If the maker of Roquefort cheese will inoculate properly with *Streptococcus lacticus* and *Penicillium roqueforti*, and provide the proper condition of manufacture and ripening, he need have no other concern about biological ripening agents.

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A STUDY OF THE STREPTOCOCCI CONCERNED IN CHEESE RIPENING

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INTRODUCTION

In a general way streptococci may be divided into three groups: First, the pathogenic streptococci, which differ among themselves in virulence and in their predilection for certain organs, as well as in certain biochemical reactions. Second, those streptococci which are common in the udder, in the saliva, and in the intestines. These, so far as we know, differ from the first group chiefly in their lack of virulence. Third, the milk-souring streptococci (*Streptococcus lacticus*).

It is the purpose of this paper to present streptococci in another rôle—viz, as the producers of flavor substances and other ripening changes in food prepared for consumption by fermentation, and particularly in the ripening of cheese. In this connection the streptococci are important as one of the factors concerned in rendering palatable many articles of food in use throughout the world. But they are unobtrusive, and demand little attention—only that the proper conditions for their growth shall be provided. The manufacturer of the food meets this demand without knowing why; he only knows that if he follows such and such a procedure he will obtain the desired results. Therefore the flavor-producing streptococci have escaped notice.

Streptococci from all four of the sources mentioned differ so slightly that there has been much discussion as to whether they are varieties of the same species. Several species, however, all belonging to the genus Streptococcus, have come to be generally recognized. The accepted definition of the genus as given by the Winslows (*23, p. 141*)[2] is as follows:

> Parasites. Cells normally in short or long chains (under unfavorable cultural conditions, sometimes in pairs and small groups, never in large packets). Generally stain by Gram. On agar streak, effused translucent growth, often with isolated colonies. In stab culture, little surface growth. Sugars fermented with formation of large amount of acid. Generally fail to liquefy gelatin or reduce nitrates.

[1] The writer is indebted to her collaborators in the Dairy Division for hearty cooperation whenever the investigation was found to extend into the special field of research of any one of them. The gas analyses were made by Dr. W. M. Clark; Dr. J. N. Currie made the volatile-acid determinations; Mr. H. C. Jordan made the experimental cheese of the Cheddar type; and Mr. K. J. Matheson and Mr. F. R. Cammack made the soft-cream cheese.

[2] Reference is made by number (italic) to "Literature cited," p. 251–252.

The streptococci concerned in cheese ripening, *S. lacticus* and the other varieties described in this paper, comply with the generic description in every detail except in regard to parasitism. The cultural characteristics of the group are too well known to be described further in this paper, which has to do with a classification of those streptococci that act as ripening agents in cheese and other food substances.

Streptococci agreeing with the genus description given above, and differentiated from *S. lacticus* by their growth in milk culture, have been isolated from many kinds of cheese by several investigators. As the data to be presented will show, the manner of growth in litmus milk is a reliable characteristic for the differentiation of *S. lacticus* from other distinct varieties of streptococci which are normal inhabitants of various kinds of cheese. Therefore it may be safely assumed that the investigators in question were dealing with varieties of streptococci other than *S. lacticus*. For convenience in discussion in this paper, all other types of streptococci as distinguished from *S. lacticus* will be called "cheese streptococci." Henrici (*11*) found them in Schweitzer, Edam, Gouda, Port du Salut, American, Lauterbacher, Monsheimer, Münster, Limburg, Spunden, Schloss, Cantal, Brie, cream, and Neufchâtel cheeses. Troili-Petersson (*21*) found them in what the Germans call Swedish Güterkäse. Eldredge and Rogers (*4*) found streptococci in Emmental cheese. Evans, Hastings, and Hart (*8*) reported that they are active agents in the ripening of Cheddar cheese. In an accompanying paper (*7*) they are shown to be one of the predominating types of bacteria in Roquefort cheese.

Thus, it has been shown that cheese streptococci are present in both hard and soft cheeses made by various methods in different countries. Freudenreich (*9*) found streptococci that were identical with one of the varieties described in this paper to be active in the preparation of "*kefir*," and Saito (*17*) found that these same organisms were active in the fermentation of the mash from which the Japanese condiment "*soya*" is prepared. The writer has found similar streptococci in the Chinese "*toku*," or soybean cheese. It is probable that they are active in the fermentation of other foods.

The source of the cheese streptococci is suggested by the fact that other bacteria concerned in cheese making and cheese ripening, *Bacterium bulgaricum* and *S. lacticus*, are normal inhabitants of the intestines and saliva of animals. Some strains of the cheese streptococci agree in every detail with the published descriptions of *S. mitis;* others agree with the descriptions of *S. faecalis*, both of which are streptococci normally inhabiting the mouth and intestines of men and domestic animals. Other cheese streptococci differ from the species mentioned only in fermenting slightly different combinations of test substances; hence, it appears that the cheese streptococci get into the milk with the filth of the stable. Those which multiply in cheese must be particularly hardy

strains, for they develop in the presence of the by-products of *S. lacticus* and other cheese organisms; in certain types of cheese they develop in the presence of a high concentration of sodium chlorid (10 per cent in Roquefort cheese); in the manufacture of other types of cheese they survive the "cooking" of the curd (from 52° to 60° C. for about an hour in the making of Emmental cheese).

The tests ordinarily used for the differentiation of streptococci reveal nothing peculiar about the cheese streptococci. After applying the tests, therefore, it was necessary to study the cultures biochemically along such lines as observation of their conduct pointed out to be possibly fruitful. The result has been the recognition of two distinct species of cheese streptococci in addition to *S. lacticus*. Having recognized the species, and knowing something of their food requirements, the way is opened to a chemical investigation of their by-products which should yield interesting results. This paper presents the bacteriological differentiation between *S. lacticus* and the two other species, with a few fundamental facts about their peculiar physiological activities and the relation of the organisms to cheese ripening.

METHODS OF STUDY

All cultures were incubated at 30° C. For the study of morphology smears were made from 24-hour cultures from the condensation water of agar slopes. The smears were stained according to the Gram-Weigert method, and drawings were made with the aid of the camera lucida.

The methods for determining the liquefaction of gelatin, decomposition of asparagin and urea, and reduction of nitrate are described in a previous publication (*6*) to which the reader is referred. All cultures studied reacted negatively to these tests, and therefore they will not be mentioned again. The fermentation reactions were determined in the following test substances: Dextrose, lactose, saccharose, raffinose, salicin, mannite, inulin, and glycerin. Every strain reacted positively to dextrose and negatively to inulin. These substances therefore had no differential value and will not be mentioned again. One per cent of the test substance was added to a yeast-peptone broth made of 10 gm. of peptone, 5 gm. of desiccated yeast, and 5 gm. of dibasic potassium acid phosphate in a liter of water. Desiccated yeast was used in place of the usual meat extract because it is in general use in these laboratories and not because it was peculiarly adapted to the problem at hand. Sodium hydroxid was added to bring the medium to a hydrogen-ion concentration of $P_H = 6.8$. The cultures were incubated for seven days.

The hydrogen-ion concentration was then determined by the colorimetric method, with the standard solutions described by Clark and Lubs (*1*) and the indicators described by them (*14*).

The same yeast broth, without addition of a fermentable substance, and without the addition of sodium hydroxid, was found to be a very

useful medium for the study of the streptococci. The hydrogen-ion concentration of such medium is about $P_H = 6.0$.

Cultural characteristics were determined in litmus skim milk, and in litmus skim milk to which 0.5 per cent of peptone had been added.

Another very useful medium was trypsin-digested milk. One per cent of commercial trypsin was added to the milk, and it was kept at about 40° C. for two or three hours. Sodium hydroxid was added to bring the reaction to about $P_H = 7.2$ at the beginning of the digestion and several times afterward to neutralize the liberated amino acids. The final hydrogen-ion concentration of the medium was about $P_H = 6.2$. The medium was filtered, heated in the autoclave as for sterilization, refiltered, put into tubes or flasks and sterilized for use.

Volatile-acid production was determined in milk cultures. Most of the cultures were grown in 500 c. c. of skim milk, but a few of the first determinations were made in cultures grown in 200 c. c. of skim milk. For these the data have been calculated for 500 c. c. Duplicate cultures were analyzed after a varying length of incubation, and the results showed that after the first week there is little or no increase in volatile acids. The subsequent determinations were made after from 7 to 15 days' incubation. Dilute phosphoric acid was added to the culture until a blue color was given with Congo red, to release any volatile acid that might be in combination. In some preliminary determinations sulphuric acid had been employed for the purpose, but it was decided that small quantities of formic acid resulted during the distillation from the action of sulphuric acid on some constituent of the milk, possibly casein. The cultures were distilled with steam until 2,000 c. c. of distillate were collected. At first an effort was made to distill under reduced pressure to hasten the process, but it had to be abandoned because the cultures foamed so freely.

The distillate was neutralized with barium hydroxid [$Ba(OH)_2$], evaporated to a small volume, decomposed with sulphuric acid (H_2SO_4) and distilled by the method of Duclaux (*3*). This method serves both to identify and to estimate the volatile acids present. Although some of the data indicated traces of acids of lower molecular weight than acetic, probably formic, and also traces of acids higher than acetic, possibly butyric, which might arise from a slight hydrolysis of the fat in the milk, it seems justifiable to conclude that acetic acid is the only volatile acid produced by either *S. lacticus* or the cheese streptococci in their normal processes of metabolism in milk culture.

STREPTOCOCCUS LACTICUS

This organism has been known in the literature under the names "*Bacterium güntheri*," "*Bacterium lactis acidi*," and various other names, but is now generally designated as "*Streptococcus lacticus* (Kruse)." Its close relationship to the other varieties of streptococci argues forcibly

against classifying it with the rod forms, as the generic name "Bacterium" implies. Undoubtedly some authors have included under the name "*Streptococcus lacticus*" the varieties of streptococci which the results of this study show to be quite distinct.

S. lacticus has a tendency to form elongated cells with pointed ends, and to separate in pairs rather than remain together in long chains. These characters are variable, however, according to the conditions under which the organism is grown. The other cheese streptococci also form elongated cells under certain conditions, and some strains of them are no more inclined to form chains than is *S. lacticus;* hence, morphology will not distinguish *S. lacticus*.

A summarized description of the *S. lacticus* group is given in Table I. The cultures included in the table were isolated from various sources. Culture 96mz was isolated from a commercial starter. Culture 96ga was from an experimental Roquefort cheese, and is probably the same strain as culture 96mz, the starter culture. Culture 2ak was from unpasteurized milk. Cultures 96hr and 96hs were from cream from two dairies. Cultures 2am, 2al, 2ao, 2an, and 2ap were from pasteurized milk from five dairies. Culture 2ab was from Cheddar cheese. Culture 96ht was from soybean cheese imported from China.

The growth of *S. lacticus* in litmus milk is characteristic. This streptococcus begins to reduce the litmus before the reddening appears, and before the milk coagulates, the reduction is complete beneath the pink surface layer. Esten (*5*) noted this reaction. Sherman and Albus (*19*) have recently confirmed it as a typical reaction for *S. lacticus*, although other types of bacteria also give the same reaction. The data to be presented in this paper show that the characteristic reduction of litmus, which is correlated with other physiological activities, serves well in routine work for a differentiation of it from other varieties of cheese streptococci. In an active culture of *S. lacticus* curdling takes place in less than 24 hours. Weakened strains require a longer time, or they may fail to curdle the milk.

Another characteristic of *S. lacticus* in milk cultures is the formation of crystals.[1] White specks or crystals are familiar in well-ripened Cheddar cheese, and occur also in well-ripened Roquefort cheese. Dox (*2*) identified the crystals from Roquefort cheese as tyrosin. Crystals isolated from Cheddar cheese and from pure-milk cultures of streptococci have been identified as calcium compound of an amino acid. Crystals were formed by all but 1 of the 12 strains of *S. lacticus* which served for this study.

In one of the earliest descriptions of *S. lacticus*, or "*Bacterium lactis acidi*," as he called it, Leichmann (*13*) reported that it produced lactic acid with no volatile acid. Jensen (*12*) and others have since shown

[1] For the determination of crystal formation milk cultures are covered tightly with tin foil over the cotton plug and incubated for six weeks or longer.

that small quantities of volatile acids, chiefly acetic, were produced by this organism. In an earlier work (*10*) it was shown that some strains of streptococci which did not give a reduction of litmus characteristic of *S. lacticus* produced large quantities of acetic acid in milk cultures. This suggested that differences in acetic-acid production might serve as a basis for the differentiation of cheese streptococci. Therefore volatile-acid determinations were made on milk cultures of five strains of *S. lacticus*, four of which produced between 9.90 and 11.27 c. c. *N*/10 acetic acid in 500 c. c. of milk. The other strain produced 7.25 c. c. of acetic acid. Cultures of *S. lacticus* agree, therefore, in producing a small and fairly constant quantity of acetic acid in milk cultures, equivalent to about 0.12 gm., calculated for 1 liter of milk.

Jensen (*12*) found that *S. lacticus* could develop in peptone broth without any carbohydrate, and that when growing thus it was able to decompose the peptone with the formation of amino acids and ammonia. This is a definite reaction when measured in terms of the hydrogen-ion concentration. Cultures of *S. lacticus* inoculated into carbohydrate-free yeast peptone broth with a hydrogen-ion concentration of $P_H = 6.0$ grew abundantly, reducing the hydrogen-ion concentration to $P_H = 6.8$. This indicates a vigorous proteolytic activity. Two cultures varied slightly from this final hydrogen-ion concentration; and two cultures, 96ga and 2am were atypical in failing to grow in the carbohydrate-free medium.

S. lacticus cultures isolated from Cheddar cheese were reported in an earlier work (*8*) to be positive in their fermentation of lactose in broth cultures, negative in glycerin broth, and either positive or negative in salicin, mannite, and sucrose broth. The fermentation reactions of the *S. lacticus* cultures isolated for the present study agreed with the earlier description. The order of availability of the test substances was the same as noted in the earlier publication—viz, lactose, salicin, mannite, sucrose. Glycerin and raffinose were not fermented. The fermentation reactions of each of the 12 cultures which were studied in detail ware presented in Table I. The final hydrogen-ion concentrations are given only for those cultures which hydrolyzed the test substance. The minus sign (−) does not indicate a failure to grow, but a failure to form acid. As would be expected, if the test substance is not attacked, the same reaction would take place as in the plain-yeast-peptone broth. The table shows that there is a considerable variation in the final hydrogen-ion concentration of any one strain in the different broths, and also that there is a variation in the final hydrogen-ion concentration of the different strains in any one of the broths. The final hydrogen-ion concentrations varied from $P_H = 4.0$ to $P_H = 5.0$. The same was the case in dextrose broth, not included in the table. The succeeding tables show that the same thing is true of the other cheese streptococci, and that the different species cover the same range of variation. Therefore the determination of the final

hydrogen-ion concentration in the broth medium used in this study does not aid in the classification of the streptococci under consideration. It may be possible to devise a medium in which the final hydrogen-ion concentration of the different varieties would be characteristic.

TABLE I.—*Characteristics of Streptococcus lacticus*

Culture No.	Changes in litmus milk.		Crystals in milk.	Quantity of N/10 acetic acid in 500 c. c. of milk.	Final P_H values in peptone-yeast broth (P_H=6.0).	Final P_H values in fermented broths.[a]					
	Curdling.	Reduction.				Lactose.	Salicin.	Mannite.	Sucrose.	Raffinose.	Glycerin.
	Days.			*C. c.*							
96mz	2	Complete	−	7.25	.....	5.0	−	−	−	.....	−
96ga	2	...do.....	+	9.90	No growth.	4.1	−	−	−	−	−
2am	1	...do.....	+	.....	...do...	4.7	−	−	−	−	−
2ak	1	...do.....	+	.....	6.8	4.6	4.7	−	−	−	−
96hr	1	...do.....	+	11.27	6.8	5.0	5.2	−	−	−	−
96hs	1	...do.....	+	10.65	.....	5.0	5.2	−	−	−	−
2al	2	...do.....	+	.....	6.8	4.7	4.7	4.9	−	−	−
2ao	1	...do.....	+	.....	6.8	4.5	4.7	4.8	−	−	−
2an	1	...do.....	+	.....	6.6	4.6	4.6	−	4.2	−	−
2ab	1	...do.....	+	10.56	6.8	4.6	4.7	4.6	4.1	−	−
96ht	2	...do.....	+	.....	6.9	4.6	4.6	4.6	4.4	−	−
2ap	1	...do.....	+	.....	6.8	4.6	4.6	4.8	4.4	−	−

[a] The minus sign (−) indicates failure to form acid.

STREPTOCOCCUS X

It was stated above that after applying to all the streptococcus cultures isolated from cheese the biochemical tests which served to characterize *S. lacticus*, two other species were recognized. One of them is designated *Streptococcus X*, which is merely a tentative appellation to serve until a better knowledge of the milk and cheese streptococci is at hand. The relation of this group of organisms to cheese ripening will be discussed later in this paper. To anticipate that discussion it may be stated here that *Streptococcus X* brings about changes in ripening cheese which are easily distinguishable from the changes caused by *S. lacticus*. This is one of the most forceful arguments to justify considering it a species distinct from *S. lacticus*.

Morphologically *Streptococcus X* differs slightly from *S. lacticus*. In the condensation water from agar slopes the cells occur commonly in pairs and under some conditions the cells are elongated and slightly pointed at the ends. But *Streptococcus X* has more of a tendency than *S. lacticus* to form groups of from two to seven cells. This grouping of cells often suggests that they may belong to the micrococci rather than to the streptococci, but short chains of from four to eight cells are to be found in almost every culture. In dextrose broth long chains are formed, which classifies the group definitely with the streptococci.

Streptococcus X was found to be abundant in Cheddar and Roquefort cheese. Thirteen representative strains were selected for detailed study. Four of them were from Cheddar, and nine were from imported and experimental Roquefort cheese. In Table II, where the data for this group are summarized, the strains numbered 2 were from Cheddar cheese, and those numbered 96 were from Roquefort cheese.

TABLE II.—*Characteristics of Streptococcus X*

Culture No.	Changes in litmus milk.		Crystals in milk.	Quantity of *N*/10 acetic acid in 500 c. c. of milk.	Final P_H values in peptone yeast broth (P_H=6.0).	Final P_H values in fermented broths.[a]					
	Curdling.	Reduction.				Lactose.	Salicin.	Glycerin.	Mannite.	Sucrose.	Raffinose.
	Days.			*C. c.*							
2ac	4	None	—	49.39	6.8	4.6	4.7	—	5.2	—	—
2ae	4	do	—	67.75	6.8	4.8	4.0	—	—	4.0	—
2aa	5	do	—	70.00	6.8	4.5	4.2	6.1	—	—	—
96ab	(*b*)	do	—	.....	6.7	3.8	5.8	5.9	—	—	—
96et	6	Partial	—	63.76	6.8	4.7	4.8	6.1	—	—	—
96hb	12	None	—	62.34	6.8	4.7	4.7	6.5	—	—	—
2ai	5	Slight	—	60.12	6.6	5.1	5.4	6.5	—	—	—
96dc	6	Partial	—	65.50	6.8	4.6	4.7	5.4	4.9	—	—
96hi	3	Slight	—	.....	6.8	4.0	4.5	5.6	4.8	—	—
96bu	5	do	—	58.25	6.8	4.0	4.1	5.6	0	3.8	—
96fh	2	None	—	.....	6.8	4.6	4.3	6.2	0	4.3	—
96dq	6	Slight	—	57.88	6.8	4.5	4.5	5.2	5.2	4.2	—
96gx	3	do	—	65.24	6.8	4.2	4.7	6.4	5.1	5.2	—

[a] The minus sign (—) indicates failure to form acids. [b] Not curdled in 14 days.

Streptococcus X requires a longer time than *S. lacticus* to curdle litmus milk. Table II shows that the cultures varied in that respect from 2 to 12 days. There is a partial reduction, or no reduction of the litmus. In peptone milk curdling usually takes place in one day. There is no crystal formation in milk cultures.

The most striking differentiation between *S. lacticus* and *Streptococcus X* is the large quantity of acetic acid formed in milk cultures by the latter. The quantity of acetic acid produced in milk cultures was determined for 10 strains of this group. With the exception of one culture, 2ac, which produced 49.39 c. c. *N*/10 acetic acid in 500 c. c. of milk, which was somewhat less than was characteristic for the group, the quantity was fairly constant, varying in eight cultures between 57.88 and 67.75 c. c. Culture 2ac produced 70 c. c., slightly more than the characteristic quantity of acetic acid. If calculated for 1 liter of milk, the characteristic quantity of acetic acid produced by this group of streptococci is equivalent to 0.7 to 0.8 gm. per liter, or approximately six times the quantity of acetic acid produced by *S. lacticus*.

The reduction of the hydrogen-ion concentration in peptone-yeast broth with an initial concentration of $P_H = 6.0$ was precisely the same for *Streptococcus X* as for *S. lacticus*. Two of the thirteen cultures varied slightly from the characteristic final $P_H = 6.8$.

The order of availability of the fermentable test substances is somewhat different from that for *S. lacticus*. In both groups lactose is fermented by every strain. The fermentation tests were made for 27 strains of *Streptococcus X* in addition to the 13 strains included in the table. Every one of the 40 strains fermented salicin. All but 2 of the strains, 2ac and 2ae (Table II), or 95 per cent, fermented glycerin; 27, or 67.5 per cent, fermented mannite; and 14, or 35 per cent, fermented sucrose. The order of availability of the test substances of *Streptococcus X* is, therefore, lactose and salicin, glycerin, mannite, sucrose. The high percentage of positive reactions in glycerin broth characterizes this group as compared with *S. lacticus*. Raffinose was not fermented.

Under the conditions of the tests, the final hydrogen-ion concentrations in fermented broths varied widely, and failed to characterize the group. In glycerin broth the final hydrogen-ion concentration was notably less than in the other fermented broths.

STREPTOCOCCUS KEFIR

This organism was first isolated by Freudenreich (*9*), who called it "*Streptococcus b.*" Migula (*15*) gave it the name "*Streptococcus kefir.*" Under the name "*Bacterium soya,*" Saito (*17*) described an organism which is apparently identical with this one. Out of consideration for priority in names, this group of organisms should be called "*Streptococcus kefir.*"

There is nothing characteristic about the morphology of *S. kefir*. In the condensation water from agar slopes it occurs in pairs, and occasionally short chains are formed. It has not so pronounced a tendency to group formation as *Streptococcus X*, but occasionally groups of from four to six cells are to be found.

Freudenreich found *S. kefir* to be a predominating organism in kefir. Saito found it to be one of the organisms concerned in the fermentation of the mash in the preparation of the Japanese condiment *soya*. It has been found in very great numbers in Roquefort and Cheddar cheese. No doubt a further search for this organism will show that it is important in the fermentation of other kinds of food substances, as well as other kinds of cheese. In Table III, where the data for this group are summarized, the strains numbered 96 are from imported and experimental Roqufort cheese, and the strain numbered 2 is from Cheddar cheese.

In litmus milk growth is slow, and the milk is rarely brought to curdling. Usually the acidity is only slight. There is no reduction of litmus. In peptone milk the behavior of *S. kefir* is striking and distinguishes it from all other known streptococci of food and dairy products. The peptone milk is acidified rapidly, with curdling in from three to six days. The curd is rent with escaping gas, and gas bubbles can be seen rising in rapid succession.

TABLE III.—*Characteristics of Streptococcus kefir*

Culture No.	Changes in litmus milk.		Changes in peptone milk.		Behavior in peptone yeast broth (P_H= 6.0).	Final P_H values in fermented broths.[a]					
	Acidity.	Reduction.	Curdling.	Gas.		Lactose.	Sucrose.	Raffinose.	Salicin.	Mannite.	Glycerin.
			Days.								
96gq...	Slight.....	None.	3	+	No growth.	4.0	4.6	—	—	—	—
2aj.....	...do.....	...do..	4	+	...do...	5.0	5.2	—	—	—	—
96aw..	Not curdled.	...do..	3	+	...do...	4.8	4.1	6.4	—	—	—
96ew..	Slight.....	...do..	6	+	...do...	5.4	4.7	5.3	—	—	—
96fi....	Not curdled.	...do..	2	+	...do...	4.8	4.2	4.9	—	—	—
96dd...	Slight.....	...do..	3	+	...do...	4.0	4.3	6.0	4.5	—	—

[a] The minus sign (—) indicates failure to form acids.

The best medium found for the growth of *S. kefir* is digested milk, in which there is a vigorous gas production. Quantitative and qualitative gas analyses were made according to the method described by Rogers, Clark, and Davis (*16*). In 20 c. c. of the digested milk, with an initial hydrogen-ion concentration of P_H=6.6, 26.80 c. c. of gas were produced by culture 96fi. This culture produced only 3.82 c. c. of gas in 20 c. c. of dextrose-yeast broth, and 2.82 c. c. of gas was produced in 20 c. c. of a lactose-yeast broth culture of this same strain. The growth in the sugar broths was abundant, with a vigorous acid formation. In all these media the gas produced was pure carbon dioxid, with only a trace of some other gas, undoubtedly due to experimental error. The source of the carbon dioxid has not yet been determined.

The gas production of this group of cheese streptococci served to identify it with the organisms described by Freudenreich (*9*) and Saito (*17*). Freudenreich reported that his streptococcus produced 12 c. c. of gas in 50 c. c. of a lactose-peptone broth; lactic acid also was formed. Saito reported that his organism produced carbon dioxid, lactic acid, and alcohols.

Culture 96gq of *S. kefir* grown in 500 c. c. of skim milk produced 57.14 c. c. of *N*/10 acetic acid. The milk had not been acidified to the point of curdling. In 500 c. c. of a milk culture to which 0.5 per cent of peptone had been added culture 96gq produced 80.50 c. c. of *N*/10 acetic acid, equivalent to 0.97 gm. of acetic acid per liter.

S. kefir will not grow in peptone-yeast broth without a carbohydrate. Lactose and sucrose were fermented by every one of the 23 strains on which the tests were made. Raffinose was fermented by most of the strains. Salicin was fermented by only one strain, culture 96dd. The order of availability of the test substances for *S. kefir*, therefore, is as follows: Lactose and sucrose, raffinose, salicin. Mannite and glycerin are not fermented. To judge from the limited number of cheese streptococci which have been studied, it appears that the fermentation of lactose and

sucrose with failure to ferment mannite and glycerin characterizes a cheese streptococcus as a gas producer, for no strain which has fermented this combination of test substances has failed to give a gas production in peptone milk. The fermentation of raffinose by most strains also distinguishes *S. kefir* from *S. lacticus* and *Streptococcus X*.

COMPARISON OF S. LACTICUS, STREPTOCOCCUS X, AND S. KEFIR

S. lacticus, *Streptococcus X*, and *S. kefir* are three well-defined species. *S. lacticus* is characterized by a vigorous reduction of the litmus in milk cultures, which precedes the curdling, and is complete beneath the pink surface layer; about 0.12 gm. of acetic acid is produced per liter of milk; crystals are formed in milk cultures by a large percentage of the strains; and there is a vigorous production of alkali by the majority of strains grown in peptone broth free of fermentable substance. *Streptococcus X* is characterized by a slower production of acidity in milk cultures, with only a partial reduction of the litmus; no crystals are formed in milk cultures; about 0.75 gm. of acetic acid is produced per liter of milk; and all strains produce alkali in peptone broth. *S. kefir* is characterized by a faint growth in milk cultures; no growth takes place in peptone broth; the acetic acid production is similar to that of *Streptococcus X;* and there is a vigorous production of gas in peptone milk or digested milk cultures.

A summarized description of the three species of streptococci is given in Table IV, where the characteristic reactions can be compared. Average values are given in those columns of the table where the test is expressed in numbers. Considering all the tests, the fermentation reactions gave the least aid in this classification. The order of availability, however, is distinctly different for each species, and there are certain individual reactions which are more or less characteristic. For example, fermentation of glycerin would indicate that a streptococcus belonged to the *Streptococcus X* group; fermentation of raffinose would indicate that a strain belonged to the *S. kefir* group; fermentation of sucrose is also somewhat characteristic of *S. kefir*, for it has been fermented by every strain of this group, whereas the majority of strains of the other two groups failed to ferment sucrose.

TABLE IV.—*Comparative description of S. lacticus, Streptococcus X, and S. kefir*

Organism.	Changes in litmus milk.		Crystals in milk.	Changes in peptone milk.		Quantity of N/10 acetic acid in 500 c. c. of milk.	Final P_H values in peptone yeast broth (P_H=6.0).	Fermentation of [a]—					
	Curdling.	Reduction.		Curdling.	Gas.			Lactose.	Salicin.	Mannite.	Sucrose.	Raffinose.	Glycerin.
				Days.		*C. c.*							
Streptococcus lacticus.	1 day.....	Complete	±	1	−	10.00	6.8	+	±	±	±	−	−
Streptococcus X...	5 days.....	Partial..	−	1	−	62.00	6.8	+	+	±	±	−	±
Streptococcus kefir	Slight acidity.	None....	−	3	+	[b] 80.00	No growth	+	±	−	+	±	−

[a] The minus sign (−) indicates failure to form acids. [b] Peptone was added to the milk.

OTHER STRAINS

Not all the cheese streptococci can be classified in one or another of the three groups which are described above, although most of the strains which were studied agreed in every detail with those types. The irregular strains will not be described in this paper for lack of sufficient data. The study of a larger number of strains of streptococci from a wider variety of fermented foods will most probably show that there are other distinct types of flavor-producing streptococci, to which some of these irregular strains may belong. Others are probably atypical strains.

A few strains isolated from Roquefort cheese were non-lactose-fermenting and agreed in every way with the descriptions of *S. equinus*, but they have not been encountered frequently enough to suggest that they may be of importance in cheese ripening.

STREPTOCOCCUS X AND S. KEFIR AS RIPENING AGENTS OF CHEDDAR CHEESE

As a result of an earlier work on Chedder cheese (*8*), the conclusion was drawn that this type of cheese could not ripen normally without the activity of several strains of streptococci. When the milk was pasteurized for cheese making, the streptococcic flora consisted entirely of *S. lacticus* used as a starter. Such a cheese never developed the delicate flavor typical of the Cheddar type, but instead it retained the acid flavor of the curd until the influence of *Bacterium bulgaricum* became evident in the late stages of ripening. The texture of the pasteurized-milk cheese was also unlike that of raw-milk cheese. The pasteurized-milk cheese remained white, opaque, and somewhat soggy, whereas the raw-milk cheese became almost translucent, and developed a yellowish color. The addition of one other strain of cheese streptococcus, together with *S. lacticus* in the starter, modified the pasteurized-milk cheese favorably; but the typical Cheddar flavor was never obtained in the earlier series of experiments. It was shown that several varieties of streptococci could be isolated from a ripening Cheddar cheese made from raw milk, and it appeared that the several varieties were necessary to produce typical flavors.

Two species of cheese streptococci have now been differentiated from *S. lacticus*. A second series of experimental pasteurized cheese has been made to show the effect of *Streptococcus X* and *S. kefir* on the ripening process, with definite results. The studies of only three of the cheeses need be mentioned to illustrate these results. The milk was pasteurized for all three cheeses. One cheese was inoculated with a starter of *S. lacticus*. It developed the acid flavor, and retained the white, opaque, somewhat soggy curd characteristic of a cheese made in this manner. The second cheese was made in just the same way, but had added to it a starter of *Streptococcus X* in addition to the *S. lacticus* starter. This cheese was decidedly milder in flavor, the mildness being apparent as a

lesser acidity. In color and texture it was noticeably more like a raw-milk cheese after about two months of ripening. Samples were submitted to several persons, who agreed that the experimentally inoculated cheese was preferable to the control. The third cheese was made in the same way, but had starters of *Streptococcus X* and *S. kefir* in addition to the *S. lacticus* starter. After two months' ripening it was unquestionably the best cheese of the three. In fact, a Cheddar cheese can rarely be obtained in market altogether as pleasing as this cheese because the undesirable organisms in milk when it comes to the factory commonly give to cheese "unclean" flavors. In the pasteurized-milk cheese all undesirable organisms have been destroyed, and in the case of the cheese in question, a part, at least, of the flora necessary for developing the delicate flavors typical of a Cheddar cheese made from raw milk had been restored. For a few weeks during the third and fourth months of ripening the flavor of the one inoculated with cheese streptococci did not compare so favorably with that inoculated with *S. lacticus* alone. After four months' ripening at about 23° C. the cheeses were placed in a refrigerator where the temperature varied from 12° to 15° C. When they were seven months old the cheese inoculated with *Streptococcus X* and *S. kefir* was pronounced by experts to be a good Cheddar cheese, with the typical flavor highly developed, whereas the control cheese inoculated with *S. lacticus* alone had a mild, rather pleasing flavor, which did not in any way resemble Cheddar.

In the last series of experiments 10 cheeses were made. One control was made of raw milk with a starter of *S. lacticus*, three were made of pasteurized milk with a starter of *S. lacticus*, to serve also as controls; and six were made of pasteurized milk with a starter of *S. lacticus* and of cheese streptococci. Three strains of cheese streptococci were used in these experiments; one of *Streptococcus X*, one of *S. kefir*, and one whose characteristics did not conform to either of these species; it is not described in this paper because it is an odd strain in the collection of cheese streptococci. Its beneficial effect when inoculated into pasteurized-milk cheese merits for it further study and a determination of its frequency in dairy products. At the time of this writing the cheeses have been ripening for six weeks at a temperature of from 13° to 15° C. The raw-milk cheese is unquestionably the poorest of the lot. There are many small gas holes in it, and the flavor is unclean. It was made during very hot weather, and the milk was poor in quality, as is usually the case under such conditions of temperature. All three of the cheeses made of pasteurized milk and inoculated with *S. lacticus* are good in texture, and have the clean, acid flavor typical of cheeses made in this way. All three strains of cheese streptococci, when inoculated singly or in various combinations, improved the flavor of the cheese as compared with the controls. The acid flavor characteristic of the control pasteurized-milk cheese is lacking; instead there is a mild

and more delicate flavor, unanimously preferred to the acid flavor by the four cheese experts to whom the samples were submitted. The curd is also more broken down in several of the cheeses inoculated with cheese streptococci, which indicates a more rapid ripening.

The experiments have shown that the cheese streptococci improve the flavor of cheese made of pasteurized milk according to the Cheddar process. The cheeses were made on an experimental scale, where there was every facility for handling the pure cultures, but it is hoped that practical methods may be devised whereby any manufacturer can make use of these pure cultures in his factory.

Sammis and Bruhn (*18*) worked out the method by which cheese of the Cheddar type can be manufactured from pasteurized milk, and they pointed out the advantages of pasteurizing the milk for making that kind of cheese. They sent to city markets great quantities of their cheese which had been inoculated with *S. lacticus* alone. From the reports of the dealers they conclude:

> There appears to be no reason why pasteurized-milk cheese cannot be sold regularly in any market, with entire satisfaction, excepting possibly, to the limited trade that demands very high flavored cheese.

The experiments recorded in this paper show that the pasteurized-milk cheese may not only be improved in flavor by inoculation with a starter of cheese streptococci, but also the time of curing may be shortened, an important consideration with the cheese manufacturer. When the makers of Cheddar cheese recognize the advantages of pasteurizing the milk, there is no reason why a practical method may not be developed by which any manufacturer could make a choice of the strain of streptococcus or the combination of streptococci which would give a flavor he desires, and by using it constantly under uniform conditions the whole output of a factory could be made to develop its own "trade" flavor.

As a result of this and earlier (*8*) work, it may be concluded that the cheese streptococci are essential factors in normal Cheddar cheese ripening. A few fundamental biochemical reactions have been determined but not enough is known of these streptococci to explain fully the chemical processes they may be responsible for in the cheese ripening. A few facts are suggestive, however, of the part the several types of streptococci may play in the Cheddar cheese ripening. The formation of large quantities of acetic acid in milk cultures by *Streptococcus X* and *S. kefir* suggests that these organisms may be responsible for the production of alcohols and esters, substances which, according to Suzuki, Hastings, and Hart (*20*) give flavors to Cheddar cheese. They found that acetic acid formed 90 per cent of the acids obtained by the saponification of the esters and the oxidation of the alcohols of their "flavor solution" from ripe Cheddar cheese. As yet it is unknown whether the acetic acid is formed simultaneously with the lactic acid in the decomposition of the

lactose, whether it results from the decomposition of the lactic acid, or whether it comes from protein cleavage.

The vigorous production of carbon dioxid by cultures of *S. kefir* explains the hitherto unaccounted-for production of large quantities of that gas in Cheddar cheese. Van Slyke and Hart (*22*) found that a normal Cheddar cheese during 32 weeks' ripening produced carbon dioxid equal to 0.5 per cent of the fresh cheese. They found that carbon-dioxid production in the cheese continued for months after the disappearance of the lactose, and they state that apparently it came from reactions taking place in some of the amido compounds. The source of the carbon dioxid has not yet been determined, but from our results also it seems improbable that it came from a decomposition of the lactose, since a vigorous decomposition of the latter indicated by the increase in hydrogen-ion concentration takes place in peptone-broth cultures, with the production of a slight quantity of gas, whereas in digested milk an abundant gas production accompanies the lactose fermentation.

The results of cultural studies indicate that in peptone-yeast broth free of fermentable substances, the protein cleavage takes place to exactly the same extent in cultures of *S. lacticus* and *Streptococcus X*. Nevertheless the two organisms do not bring about the same changes in the casein of ripening Cheddar cheese. It has been mentioned above that the pasteurized-milk cheese ripened by *S. lacticus* alone has an acid flavor, whereas a cheese made in the same manner but with the use of a starter of cheese streptococci in addition to the *S. lacticus* starter develops a milder flavor. To judge by the palate, the difference in flavor is pronounced, but the difference in terms of hydrogen-ion concentration is slight though definite. Two pasteurized-milk Cheddar cheeses inoculated with *S. lacticus* alone after about 10 weeks' ripening had a hydrogen-ion concentration of P_H=5.7. A cheese made at the same time and inoculated with *S. lacticus* and *Streptococcus X* had a hydrogen-ion concentration of P_H=5.9, and a fourth cheese inoculated with *S. lacticus*, *Streptococcus X*, and *S. kefir* also had a hydrogen-ion concentration of P_H=5.9. This slight difference of 0.2 P_H may have been responsible for the difference in protein cleavage, which was evident in the cheeses in the white, opaque, somewhat soggy mass in the case of those inoculated with *S. lacticus* alone, as compared with a more translucent, yellowish mass in other cheeses.

CHEESE STREPTOCOCCI IN SOFT-CREAM CHEESE

The case is very much the same with soft-cream cheese as with Cheddar. Cream cheese made from the best quality of raw milk develops a mild, delicate flavor typical of this kind of cheese. Cream cheese made from pasteurized milk and inoculated with a starter of *S. lacticus* develops an agreeable acid flavor, particularly liked by many people, but on the whole is inferior to the delicate flavors developed in cream cheese made

from the best quality of raw milk. As in the case of Cheddar, cream cheese made from pasteurized milk is whiter than that made from raw milk. Anyone who has the opportunity to compare cream cheese made according to the different methods learns to distinguish them quite readily by their flavor and color. The texture also differs slightly.

Experimental soft-cream cheeses have been made from pasteurized milk with the use of *Streptococcus X* and *S. kefir* alone or together, in addition to the *S. lacticus* starter. The results were definite, showing that flavors in pasteurized-milk cheese can be influenced at will by a choice of streptococci in starters. A cream cheese inoculated with *S. lacticus* alone has its own peculiar flavor; a cream cheese inoculated with *S. lacticus* and *Streptococcus X* has a decidedly different flavor; another inoculated with *S. lacticus* and *S. kefir* has another flavor; and when all three species of streptococci are used together, still another flavor is produced. And as some people prefer their ice cream flavored with vanilla, and others like chocolate flavoring best, so the one cheese flavor is more pleasing to some people, while others prefer another. The pasteurization of the milk assures a uniform product in cheese making. This is one reason why manufacturers are loath to adopt the pasteurization of milk for cream-cheese making. They want to develop the cheese flavors which they believe to be peculiar to their own product. It may be that certain strains of streptococci have gained predominance in certain localities, so that season after season a manufacturer of cream cheese can reproduce his own peculiar cheese flavors. The experiments have demonstrated that it will be possible for a manufacturer to choose the flavor which he may want his product to develop in cream cheese made from pasteurized milk. Whether the consuming public will be discriminating enough to make the inoculation of soft-cream cheese with cheese streptococci a financial success remains to be demonstrated.

SUMMARY

(1) Streptococci that differ from *S. lacticus* are common in ripening cheese of various kinds, and in other foods prepared by fermentation. In this paper they are called cheese streptococci. It is probable that a study of the mouth, fecal, and udder types of streptococci will show that cheese streptococci belong to those familiar types.

(2) *S. lacticus* is described culturally and biochemically, and two other species of streptococci, *Streptococcus X* and *S. kefir*, are likewise described.

(3) The most pronounced biochemical characteristic which distinguishes *S. lacticus* from the other two species of streptococci described is the small quantity of acetic acid which it produces in milk cultures.

(4) *S. kefir* is notable among dairy streptococci because of its vigorous production of carbon dioxid when grown in suitable media.

(5) The experiments demonstrate that cheese streptococci modify significantly the flavor of pasteurized-milk cheese. *Streptococcus X* and *S. kefir* and another unclassified strain improved the flavor and hastened the softening of the curd of cheese made according to the Cheddar type from pasteurized milk. *S. kefir* and *Ss. X* also gave distinctive flavors to soft-cream cheese made of pasteurized milk.

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INTUMESCENCES, WITH A NOTE ON MECHANICAL INJURY AS A CAUSE OF THEIR DEVELOPMENT[1]

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INTRODUCTION

Little attention has been given by plant pathologists to the pustule-like outgrowths of tissue to which the appellation "intumescences" may be appropriately applied. The accounts of them deal mainly with descriptions of their occurrence upon the leaves, flowers, fruits, and twigs of various species of plants and with suggestions regarding the cause, in most cases not supported by experiments. Since no great imaginative power is required to see that the problem of intumescences is merely a part of the greater problem of all excrescences or overgrowths in plant and animal tissues, such as galls, tumors, knots, cankers, edemata, crowngalls, etc., intumescences then become of immediate interest to both plant and animal pathologists. While the remote or ultimate causes of these overgrowths may be entirely different, investigators of these phenomena do not appear to realize that they are probably dealing with the same proximate cause. This fact is illustrated by studies on insect galls, concerning which an enormous volume of literature has accumulated; on crowngalls, our knowledge of which is due largely to the illuminating researches of Smith (*9*, *10*, *11*)[2] and his associates; and on edemata, the cause of which is so clearly demonstrated in the brilliant experimentation of Fischer (*3*). It is the present purpose, therefore, to review briefly the more important contributions dealing with the cause of intumescences, to describe intumescences arising from mechanical injury, and to suggest that their proximate cause is the same as of edema in animals, the explanation of which appears to have been generally overlooked by plant pathologists or has not been regarded by them as applicable to overgrowths in plants.

HISTORICAL REVIEW

The designation "intumescentia" appears to have been first applied by Sorauer (*12*, *p. 222*), to pustular processes, generally yellow in color, formed upon the surface of leaves. The cells which make up these processes are always more or less enlarged. In explanation of their occurrence he states that they are brought about by such conditions as

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[2] Reference is made by number (italic) to "Literature cited," p. 238–239.

excessive moisture (*13*) or abnormally high temperature (*14*), coordinated with a reduced assimilatory activity occasioned by weak illumination. The same factors are employed by various other investigators, including Atkinson (*1*), Prillieux (*6*), Noack (*5*), Trotter (*16*), and Steiner (*15*), in explanation of the cause of intumescences.

In contrast with this explanation is that of Viala and Pacottet (*17*) and Dale (*2*), who maintain that brilliant illumination, together with a moist atmosphere, is to be regarded as the ultimate cause.

Küster (*4*) expressed the opinion that the introduction of poisonous substances may be a cause of intumescences and that they are manifestly related to insect galls. The poison for the development of these galls, he believed, was produced by the gall-forming insect. A review of literature upon gall formation, a matter beside the present purpose, and a rejection of the theory that galls result from the injection of a chemical substance by the insect, are contained in a recent paper by Rosen (*7*).

The production of intumescences upon cauliflower as a result of the stimulatory activity of copper compounds applied as sprays has been demonstrated by Von Schrenk (*8*) and has subsequently been confirmed by Rosen (*7*) and by Smith (*11*). In explaining their proximate cause, Von Schrenk maintains that the stimulatory activity is probably due to high osmotic tensions resulting from compounds of the copper salts with the protoplast.

Smith's concept of overgrowths, particularly of crowngall and malignant tumors, as expressed in recent papers (*10*, *11*), is that they are due to the removal of normal growth inhibitions. He found that various substances—namely, aldehyde, acetone, alcohol, acids, and alkalies—removed these inhibitions, not by direct chemical action, however, but by locally increasing the osmotic pressure; hence, by physical action. In the case of crowngall, various soluble substances were found to result from the metabolism of the parasite in culture. If, as he contends, these substances are slowly and continuously supplied by the intracellular growth of the organism, they would lead to osmotic disturbances which would tend to be equalized by the movement of water and dissolved foodstuffs toward the parasitized cells.

Fischer (*3*), to whom reference has previously been made, relates the cause of swellings of animal tissues which manifest themselves in states known as edema, glaucoma, and nephritis to the phenomenon of absorption both of water and of dissolved substances. The cell colloids and their state, according to this theory, determine the amount of water held by a cell, a tissue, an organ, or even the entire individual under different physiological and pathological conditions. The cause, then, of normal absorption, and consequently of abnormal absorption, resides within the cells themselves. The nature and cause of edema can most

succinctly be presented by quoting the author's terse summary, in which he says (p. 190–191):

> A state of œdema is induced whenever, in the presence of an adequate supply of water, the capacity of the colloids of the tissues for holding water is increased above that which we are pleased to call normal. Any agency capable under conditions existing in the body, of thus increasing the hydration capacity of the tissue colloids constitutes a cause of œdema. The accumulation of acids within the tissues brought about either through their abnormal production, or through the inadequate removal of such as some consider normally produced in the tissues, is chiefly responsible for this increase in the hydration capacity of the colloids, though the possibility of explaining at least some of it through the production or accumulation of substance (of the type of urea, pyridin, certain amins, etc.) which can hydrate colloids as can acids, or through the conversion of colloids having but little capacity for water into such as have a greater capacity must also be borne in mind.

DESCRIPTION OF INTUMESCENCES FOLLOWING INJURY

During the early part of June, 1917, a windstorm of sufficient velocity to break down trees of considerable size occurred in the vicinity of Raleigh, N. C. A few days later, the writer, on a field trip into the sandy lands east of the city, noted that the leaf surface of cabbage (*Brassica oleracea capitata*), particularly the lowermost leaves and the tips of the inner leaves, were covered with numerous irregularly disposed, wartlike prominences (Pl. 18, A). The individual outgrowths were hemispherical to short cylindrical in shape, yellowish to grayish in color, and projected from the surface of the leaf in a sharply defined circle. They ranged in size from mere points to structures having a diameter of 3 mm. and in extreme cases projected from the leaf surface as much as twice the thickness of the cabbage leaf. A depression corresponding to the prominence commonly appeared on the opposite leaf surface. In some places the outgrowths were so numerous as to merge into each other and consequently appeared like one large, irregular intumescence.

Microscopic examination of these intumescences shows that they begin as small swellings of the epidermis. Soon the palisade parenchyma beneath, or the spongy parenchyma, depending upon whether the intumescence originates on the upper or lower leaf surface, is involved, and the cells begin to become enlarged. At this stage the epidermis is broken or elevated on the surface of the growing projection. Some of the epidermal cells then shrivel and dry and remain as fragments on the surface of the intumescence, while others are included in the intact swollen tissues. Meanwhile the cells beneath have continued to enlarge and all of the subjacent mesophyll comes to be involved in the formation of the edematous cells. These cells are enlarged to many times their normal volume (fig. 1), are elongated, very thin-walled, and are practically destitute of chloroplasts and other protoplasmic contents. There appears to be no tendency toward suberization, as is indicated by negative tests with alkannin. The cells are not to be regarded as giant cells in

the sense in which this term is applied by animal pathologists in that they are not multinucleate, but remain uninucleate. The yellowish or whitish appearance of the protrusion is accounted for by the fact that the intercellular spaces are filled with air.

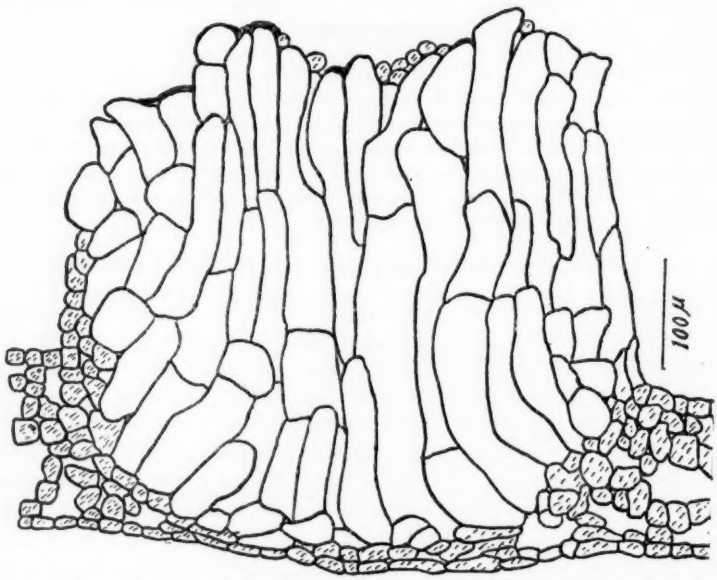

FIG. 1.—Outline drawing, made with the aid of a camera lucida, of a vertical section of an intumescence on cabbage.

Since neither fungi nor bacteria were associated with these intumescences, it was conceived that they must have resulted from injuries inflicted by wind-blown sand. This deduction was subsequently confirmed both under field and greenhouse conditions by violently projecting sand against normal cabbage plants. Intumescences of the type shown in figure B of Plate 18 developed within four to six days following the wounding. The intumescences thus artificially produced were identical in appearance and structure with those first found. They could not be made to appear upon mature parts, but only upon actively growing tissues. They occurred both on the lamina of the leaf and upon the veins. In the latter case the xylem elements were not modified, the only modification occurring in the parenchymatous cells surrounding the vascular bundles. Sections through intumescences upon small veins showed that the normal vascular elements may be entirely surrounded by hypertrophied cells.

Humidity was observed to operate in conjunction with the age of the tissues in the development of intumescences. When a plant injured by sand cast upon it was covered with a bell jar in order to maintain a high relative humidity, the edematous cells continued to enlarge until the

pustule projected from the leaf surface to a height equal to three or more times the thickness of the leaf (Pl. 19). When the enlarged cells were exposed to desiccation, however, the protrusions were much less prominent, as would be anticipated. Humidity had previously been reported by the writer as exerting a similar effect in the production of cankers on *Citrus* spp. (*18*).

DISCUSSION OF RESULTS

It must be admitted that the development of intumescences upon young cabbages following the violent projection of sand against them came somewhat as a surprise, even though wind-blown sand was suggested to the grower as the probable cause when the field diagnosis was made. Mechanical injury appears not to have been previously observed to be an ultimate cause of the formation of intumescences on plants. The complete chain of events connecting the stimulus of wounding at the one end and the mature intumescence at the other is, of course, not completely known. However, it is believed, in the light of the theory of edema as advanced by Fischer (*3*), that an adequate explanation can be offered for the formation of the enlarged cells. The exposure of the protoplasts following the rupture of the epidermis from the impact of the particles of sand would certainly be followed by oxidative changes within these protoplasts. This assumption is based on the fact that Von Schrenk (*8*) found an increase in the oxidizing enzyms in intumescences on cauliflower (*Brassica oleracea botrytis*) leaves and upon our general knowledge of oxidations following exposure of tissues through wounding. The acids produced from these oxidations would not only increase the hydration capacity of the emulsion colloids within the wounded cells but would also involve adjacent cells. The fact of a heightened affinity of colloids for water following even a slight increase in acidity is a well-known property of colloids. This would be followed by the distention of involved cells to a point where equilibrium had been established, a condition determined by such factors as differences in adsorption, solubility, and chemical combination of the cell colloids themselves.

This explanation, while it may not meet with approval by students of overgrowths, is attractive because of its simplicity and because it accords with established facts concerning absorption by hydrophylic colloids. It is furthermore believed that the theory of absorption can be applied to the experimentation presented by other investigators and can also be employed to explain adequately the mechanism of other types of overgrowths in plants such as knots, tumors, and galls of insect, nematode, bacterial, and fungus origin.

SUMMARY

(1) Various ultimate and proximate causes have been assigned for the formation of different types of overgrowths as intumescences, cankers, knots, tumors, and galls present on plant parts.

(2) Wind-blown sand as an ultimate cause of the formation of intumescences on cabbage appears not to have been previously reported.

(3) The proximate cause of these intumescences is believed to be a problem of absorption and is due to a heightened hydration capacity of the cell colloids resulting from acids produced by oxidation.

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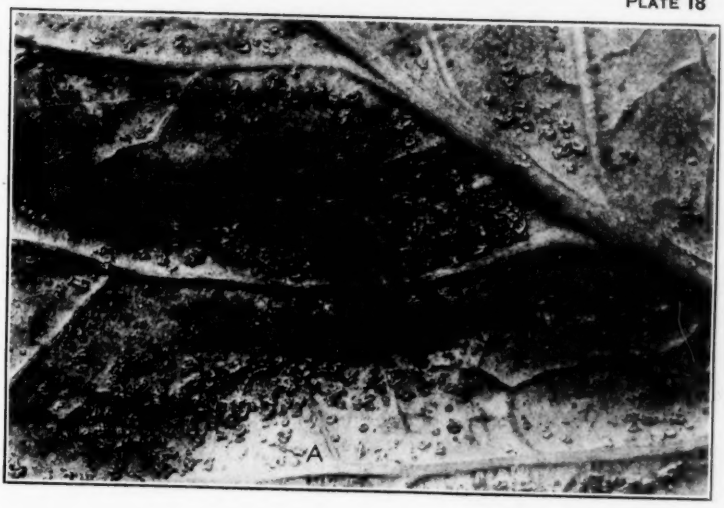
PLATE 18

A.—Intumescence on cabbage formed as a result of injury from wind-blown sand.

B.—Intumescences produced following injury from sand artificially projected against the leaves of cabbage.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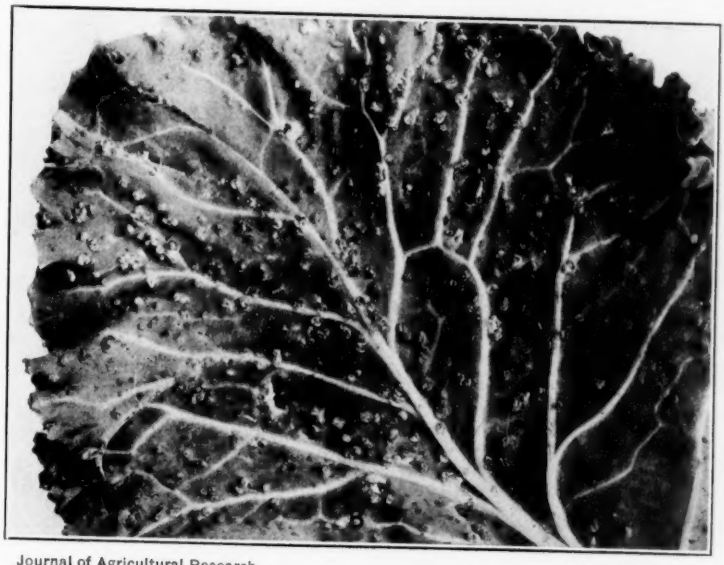

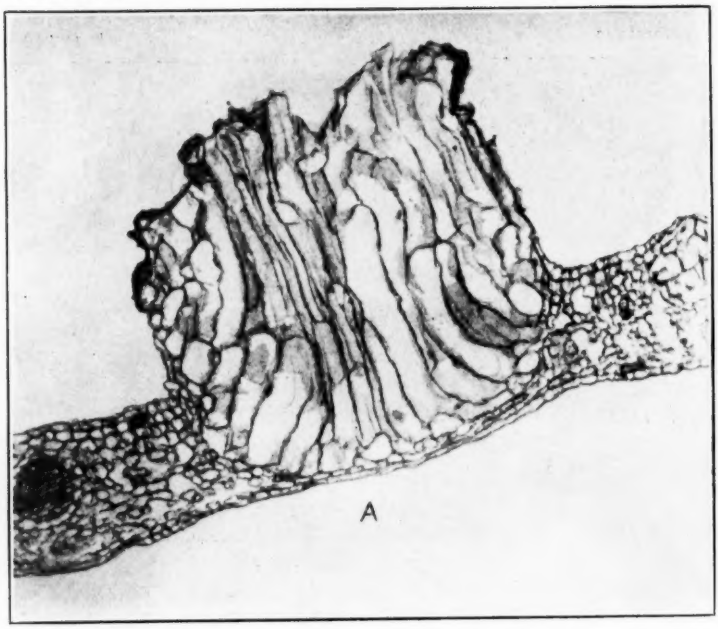
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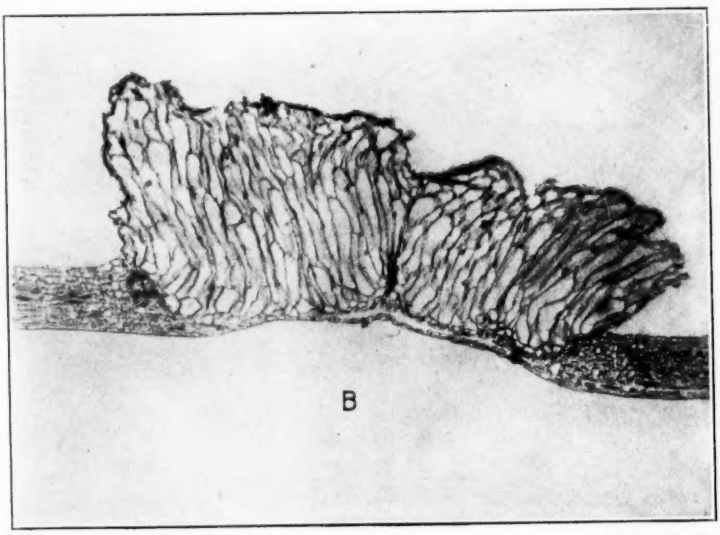
B

PLATE 19

A.—Photomicrograph of a small columnar intumescence on cabbage, following artificial injury.

B.—Photomicrograph of a large cushion-like intumescence developed after artificial injury.

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